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SPONSORED PROJECT INITIATION

Date: 7/26/80

Project Title: ~~Research Initiation~~-Determination of Microbial Flora in Potable Water

Project No: E-20-631

Project Director: Dr. Sai H. Lee

Sponsor: National Science Foundation

Agreement Period: From 5/15/80 Until ~~10/31/82~~ 10/31/83

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Date 1/28/85

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Project Director(s) Dr. Dai H. Lee GTRI / ~~GIT~~

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Title ~~Research Initiation~~ - Determination of Microbial Flora in Potable Water

Effective Completion Date: 10/31/83 (Performance) 1/31/85 (Reports)

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- ☐ None
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FINAL PROJECT REPORT
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PART I–PROJECT IDENTIFICATION INFORMATION

1. Institution and Address	2. NSF Program	3. NSF Award Number
Georgia Institute of Technology Atlanta, GA 30332	CE Research Initiation	CME-8006749
	4. Award Period From 5/15/80 To 10/31/83	5. Cumulative Award Amount $40,000

6. Project Title

Research Initiation - Determination of Microbial Flora in Potable Water

PART II–SUMMARY OF COMPLETED PROJECT *(FOR PUBLIC USE)*

The treated water contains a myriad of microorganisms that carry past the disinfection barrier and whose numbers far exceed the coliform bacteria. Presently, only a limited effort is being directed toward characterizing the microbial composition of the treated drinking waters.

The microbial flora of potable waters was investigated by focusing on these study areas: (1) determination of major microbial populations, (2) isolation and identification of microorganisms observed in enrichment culture media, (3) development of an improved procedure for bacterial plate counts, and (4) development and application of epifluorescent direct count method for the quantitative monitoring of microorganisms in potable waters. Heterotrophic aerobic and facultative anaerobic bacteria were the most predominant group of microorganisms present in drinking water. Selective enrichment culture techniques are applicable in determining the major groups of microorganisms present in potable waters. The study yielded occurrences of low-concentrations of iron-transforming, sulfate-reducing, nitrogen-fixing, nitrifying, sulfur-oxidizing, denitrifying, actinomycete and stalk/appendage-producing bacteria in water. Alternative plate count media such as R2A, m-SPC and SPC/10 were better in terms of supporting a more diverse species of heterotrophic bacteria. A new combined technique of epifluorescent direct count DAPI staining and INT reduction yielded higher and more accurate bacterial counts than the current standard plate count method.

PART III–TECHNICAL INFORMATION *(FOR PROGRAM MANAGEMENT USES)*

1. ITEM *(Check appropriate blocks)*	NONE	ATTACHED	PREVIOUSLY FURNISHED	TO BE FURNISHED SEPARATELY TO PROGRAM	
				Check (√)	Approx. Date
a. Abstracts of Theses		X			
b. Publication Citations		X			
c. Data on Scientific Collaborators		X			
d. Information on Inventions	X				
e. Technical Description of Project and Results		X			
f. Other *(specify)*					

2. Principal Investigator/Project Director Name *(Typed)*	3. Principal Investigator/Project Director Signature	4. Date
Dr. Sai H. Lee		Oct 31, 1984

Judith Lee Tender

ISOLATION AND ENUMERATION OF PHYSIOLOGICALLY DIFFERENT MICROBIAL GROUPS CHARACTERISTIC OF ATLANTA'S POTABLE WATER

ABSTRACT

The purpose of this research problem is to aid in the examination of the microbiological quality of Atlanta's potable water. Various selective enrichment culture media were used to aid in the determination of several physiological groups of organisms present in Atlanta's water distribution system.

Since Atlanta's water source is the Chattahoochee River, the various selective enrichment culture media were selected accordingly to include aerobic aquatic and soil bacteria as well as anaerobic bacteria. For each medium, the necessary chemical constituents and relative concentrations required for optimum growth were evaluated including the choice of appropriate sample volumes.

In this study the media tested were as follows: isolation medium for heterotrophic iron precipitating bacteria; filamentous iron bacteria medium; sulfur oxidizing bacteria medium; autotrophic nitrifying bacteria medium; nitrogen fixing bacteria medium; enrichment and isolation medium for stalked and budding bacteria (esp. Caulobacter and Hyphomicrobium); AGS - selective medium for aerobic actinomycetes; a nitrogen base yeast medium with glucose for molds and yeast; reinforced Baar's medium for sulfate reducing bacteria; a selective medium for denitrifying organisms; and an enrichmentmedium for anaerobic bacteria.

For the purpose of isolation and enumeration of the organisms present, the pour plate technique using solid media was employed. Of the various media tested in solid form, only the following were found to show positive growth with

Atlanta's potable water: the isolation medium for heterotrophic iron precipitating bacteria; filamentous iron bacteria medium; sulfur oxidizing bacteria medium; nitrogen fixing bacteria medium; the stalked and budding bacteria medium; and the AGS - selective medium for aerobic actinomycetes. The nitrogen base yeast medium and the isolation medium for nitrifying bacteria were applicable in solid form when hydrant samples were tested. The solid media for the enumeration of anaerobic bacteria, sulfate reducing bacteria and denitrifying organisms exhibited negative results when incubated under anaerobic conditions.

All water samples were collected from the Hemphill Water Treatment Facility distribution system. Since Atlanta's water distribution system is very large and complex, for comparative purposes water samples were collected from two distribution mains, one 20 inch diameter and the other 8 inch diameter.

The dynamic nature of the microbial flora within the distribution system was observed, for both numbers and community structures changed with time and location. Slightly higher densities of similar microorganisms were observed with water samples collected from the smaller diameter main. In addition, the overall density of organisms tended to increase as the water traveled further from the treatment plant, but the diversity of organisms did not necessarily follow the same pattern.

Andrew W. Karp

DEVELOPMENT OF AN IMPROVED PROCEDURE FOR BACTERIAL COUNTS FOR FINISHED DRINKING WATER

ABSTRACT

The objective of the project was to develop an improved bacterial enumeration technique for the analysis of bacteriological quality in finished drinking waters.

Various factors were evaluated including the plate count media, incubation temperatures, incubation times, and plating procedures. Plate count media examined were Tryptone Glucose Yeast Agar (Standard Plate Count Medium), and two other media: M-SPC and R2A developed by Taylor and Geldreich (38), and by Reasoner and Geldreich (33), respectively. A ten-fold dilution of Tryptone Glucose Yeast Agar was also examined. Three incubation temperatures, 20°C, 25°C, and 35°C, were evaluated and the bacterial counts were performed for 2 to 14 days of incubation. Various plating techniques were evaluated including the membrane filter, the spread plate, and the pour plate methods. The data indicated a better recovery of bacteria with the pour plate method than with the membrane filter method. The spread plate method was shown to be inadequate because of the very small volume that can be used with it.

Since the analysis of finished drinking waters yielded very low numbers of bacterial colonies on tested media, a modified plate count technique was developed for the analysis of such waters. The technique allowed the use of up to 30 ml of sample per plate instead of 1 ml limitation associated with the standard method. It also decreased the brief temperature shock to which the bacteria were exposed when inoculated with melted media in pour plates. The

standard procedure would expose the bacteria to a shock of about 43°C, whereas the procedure developed by the project only exposed them to about 35°C.

When densities of colony forming bacteria from the same water sample were examined, higher densities were observed in M-SPC medium, R2A medium, and the ten-fold diluted Tryptone Glucose Yeast Agar medium, than in the standard plate count medium.

The incubation time and temperature for bacterial enumeration were found to be important. Results indicated a higher density of colony forming bacteria at an incubation temperature of 25°C than at 20°C or 35°C. At 25°C, 9 days were required to provide colonies among which the majority were large enough to be sure of detection by a colony counter, and to provide statistically significant colony counts per plate.

The effect on colony formation of varying the depth of themedia was studied with three samples. The results were only tentative, due to the limited number of samples studied for this effect. It was observed that after 14 days of incubation, colony counts per ml of sample were nearly the same for media depths of 2.8 mm and 4.2 mm.

It was observed that the density of colony forming bacteria increased with sampling distance from the treatment plant, at least on the two days when this was evaluated. It was also noted that most of the pigmented colonies did not become observable until after several days of incubation. Thus the standard 2 or 3-day count did not include most of these. The majority of colonies observed over the course of the project were white, but there were many yellow, orange and pink colonies as well.

A good deal of debate regarding the significance of the total plate count was found in the literature. However, at a minimum, the plate count has served

the following two valuable purposes: (a) high plate counts correlate to interference with the test for coliform levels; and, (b) when the plate count is done on a regular basis, sudden abnormally high levels have preceded waterborne disease outbreaks, even when the coliform test failed to indicate any potential problem.

III b. Publication Citations

1. Lee, Sai H., "Application of the Epifluorescent Direct Count Method in Monitoring Bacteriological Quality of Potable Water", In the Third International Symposium on Microbiol. Ecology, East Lansing, Michigan, 1983.

2. Lee, Sai H., "Biologically Mediated Water Quality Changes in Distribution Systems", Proc. of the First Conference on Water Quality and the Public Health, Worcester, Massachusetts, 1983.

3. Lee, Sai H., "Turbidity and Microbially Mediated Corrosion", American Society of Microbiology 82nd Annual Meeting, Atlanta, Georgia, 1982.

4. Lee, Sai H., "Evaluation of Microbiological Quality of Water Supply Systems", Georgia Water Works and Pollution Control Assoc. Conference, College Park, Georgia, 1981.

III c. Data On Scientific Collaborators

Graduate Research Assistants

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Cindy Lin

PROJECT DESCRIPTION

A. Relevance of Research

Currently, the bacteriological quality of drinking water is monitored by examining the coliform bacteria in water. However, the treated water contains a myriad of microorganisms that carry past the disinfection barrier and whose numbers far exceed the coliform bacteria. Finished waters that enter a distribution network are subject to continuous bacteriological deterioration. Some factors which contribute to this deterioration include 1) inadequate dosage or maintenance of disinfectant, 2) incomplete removal of organics and/or turbidity due to treatment deficiencies, 3) seasonal temperature fluctuations, 4) extended retention periods in reservoirs, standpipes and dead-end water mains, and 5) sediment accumulations. (1) In addition, some microorganisms can also penetrate the treatment barrier during momentary lapses in treatment. Very little information is available on the microbial populations that emerge as a result of various water treatment processes including the disinfection process. When the standard plate count analysis is employed, some measurements of the general bacterial population of the treated water are performed. However, only a limited effort is being directed toward characterizing the microbial composition of the treated drinking waters.

Today both waterworks engineers and aquatic microbiologists recognize the necessity for and potential benefits of studying the microbial composition of potable water because the identification of characteristic microbial populations can yield more realistic information concerning the microbiological quality of the treated water. The delineation of both qualitative and quantitative inventories of microorganisms in potable water can yield a better assessment on

the efficiencies of various water treatment processes and water quality changes in distribution systems.

It is a fact that no single medium, temperature or choice of incubation time will insure recovery of all microorganisms present in drinking water. The major difficulty encountered in identifying the microorganisms in potable water has been the lack of available methodology to investigate the characteristic microbial populations. Many organisms observed in standard plate count media exhibit aberrant cellular morphology and uncommon physiological behavior.

Studies by Allen, et al. (1) have shown that high non-coliform populations in treated water suppressed the growth of coliform bacteria in test media. The identification of these organisms in coliform test media resulted four categories of concern: total coliform, coliform antagonists, opportunistic pathogens and a miscellaneous group of strains whose significance has not been determined.

The principal objective of this research was to evaluate both qualitative and quantitative inventories of microorganisms in potable waters. In order to carry out this objective the study focused on the following four areas with drinking waters from two water systems:

1) Determination of major microbial populations in drinking water;
2) Isolation and identification of microorganisms observed in enrichment culture media and m-Endo coliform medium;
3) Development of an improved procedure for bacterial plate counts; and
4) Development of an alternative method for the quantitative analysis of microorganisms in potable waters.

B. Background

The water cycle represents an obvious mode of transmission of enteric diseases in the community. When Pasteur and other microbiologists discovered

that microorganisms were responsible for the cause of diseases, a concept of relating bacterial densities in water to water quality was proposed in the beginning years of sanitary bacteriology, and the bacteriological examination of water became a valuable supplement to chemical analyses. In 1892, Sternberg proposed that good quality water should contain less than 100 bacteria per ml. (2) He found that the finished water having 500 organisms per ml were marginal quality and those found to contain over 1000 bacteria per ml were often contaminated with sewage or surface runoff. The relationship between the plate count and the number of pathogens that might be present in water was not specified; however, it was assumed that chance occurrences would be proportionally greater as the general bacterial population increased. Thus, for many years bacterial plate count became a standard measure of water treatment process efficiency and used as an indicator of the sanitary quality of finished waters. Then a concept was enunciated in that certain microorganisms should be identified in order to provide evidence of potentially dangerous pollution. It became clear that microorganisms examined should be originated from the excrement of man or warm-blooded animals to signify a fecal pollution. The principle of indicator organisms was established, and the coliform bacteria became the chosen group. In 1914, the analysis of coliform bacteria in water became the standard bacteriological quality control test in the United States. (3)

Currently, potable water of good bacteriological quality is maintained by the attainment of less than one coliform organism per 100 ml of water. (4) With proper treatment and an adequate disinfectant the public health risk associated organisms of sanitary significance are generally minimized. It is common knowledge that potable water contains a variety of microorganisms that are common to the finished water and whose numbers far exceed the coliform organisms. Except for those organisms that are directly associated with public health

hazards, there have been few studies conducted on the distribution of general bacterial populations in drinking waters.

In 1944, Shannon and Wallace (5) examined treated water samples from Detroit, Michigan water distribution system. They isolated 495 colonies from samples of dead-ends, consumer complaint areas and chlorinated plant effluent. There were 42 Gram positive cocci, 5 Gram positive rods and 448 Gram negative rods. These authors postulated that either these bacteria had escaped the chlorine treatment or penetrated through possible defects such as main breaks, new construction, cross-connection, etc. In the same year Alexander (6) also reported the growth of iron and sulfur bacteria as well as the following genera of bacteria in water; Bacillus, Sarcina, Micrococus, Flavobacterium, Achromobacter, Alcaligenes, Pseudomonas and Proteus. Geldreich et al. (7) conceded that as a result of inadequate treatment procedures, microbiological flora in finished water is highly variable, though the bacterial groups most frequently associated with finished water may include, among others, Pseudomonas, Flavobacterium, Achromobacter, Proteus, Klebsiella, Bacillus, Serratia, Corynebacterium, Spirillum, Clostridium, Gallionella, Arthrobacter and Leptothrix. These organisms, in general, are able to survive and many may even multiply in finished waters where nutrients are minimal. Pseudomonas and Flavobacterium are problematic genera because some species have been found to be opportunistic pathogens, i.e., they can pose a health risk to patients in hospitals, clinics, nurseries, etc. (8)

Other studies on the microbial flora of treated waters have been directed toward the identification of those organisms that are associated with consumer complaints including taste and odor, turbidity and discoloration of waters. Many investigators attributed water quality problems in distribution systems to associated infestation of various groups of microorganisms including iron

bacteria, sulfate-reducing bacteria, algae, actinomycetes, etc. (9-26) In particular, researchers like Silvey (17,21), Palmer (19) and Lin (25,26) asserted that taste and odor problems were due to the growth of organisms such as algae and actinomycetes. Larson (11) and Starkey (13) postulated that the transformation of iron was brought about by iron bacteria which have specific physiological characteristics. Considerable efforts were directed toward isolation and identification of certain iron bacteria, such as, Crenothrix, Leptothrix, Gallionella and sulfur bacteria, such as, Desulfovibrio, Thiobacillus, Beggiatoa, etc. in water systems. Since aquatic microbiologists were confronted with formidable problems of costs in studying complex systems like water supply systems, problems of water quality changes in distribution systems have been alluded to chemical analyses of treated waters. Since techniques for culturing microorganisms in water were inadequate, and water supply engineers resorted to more expedient solutions, i.e., chemical treatments, in order to alleviate water quality deterioration problems in distribution systems.

A few years ago this investigator had examined a number of water distribution systems to determine whether the water quality changes observed in distribution systems were the result of microbiologically mediated reactions. (27) Both raw and treated water samples were collected from various water supply systems, and their physical, chemical and microbiological qualities were analyzed. Results of various chemical and microbiological analyses of water samples taken from three representative water systems are shown in Tables I and II. As expected, both raw and treated water qualities of three water systems were different. In particular, they differ significantly with respect to pH, alkalinity, iron, sulfide and sulfate ions. Although it is not readily apparent from Table I, qualities of treated water samples taken at hydrant flushings were

TABLE I. CHEMICAL ANALYSIS OF RAW AND TREATED WATER SAMPLES FROM THREE WATER SUPPLY SYSTEMS IN MISSOURI

SAMPLE SOURCE	NUMBER OF SAMPLES TESTED	pH	ALKALINITY (mg/l)	TOTAL CHLORINE (mg/l)	TOTAL IRON (mg/l)	TOTAL SULFIDE (mg/l)	SULFATE (mg/l)
Water System I							
Raw Water	18	7.2-7.9[a](7.4)[b]	6.2-8.0[a](7.2)[b]	0	0.01-0.4[a](0.12)[b]	0.0-6.1[a](0.79)[b]	27-37[a](31)[b]
Consumers' Taps	26	6.9-7.9 (7.4)	5.1-8.1 (6.9)	0.0-1.2 (0.21)	0.0 -1.0 (0.18)	0.0-6.3 (1.32)	21-40 (33)
Hydrant Flushings	12	7.2-8.1 (7.8)	5.0-10.7 (7.3)	0.0-0.6 (0.12)	0.01-1.0 (0.36)	0.0-12 (2.82)	26-39 (33)
Water System II							
Raw Water	4	7.4-7.9 (7.7)	2.2-2.5 (2.3)	0	0.0 -0.8 (0.28)	0.8-2.4 (1.6)	19-29 (23)
Consumers' Taps	14	7.3-8.3 (7.8)	2.1-2.7 (2.4)	0.1-1.6 (0.58)	0.0 -0.9 (0.17)	0.2-1.6 (0.7)	23-38 (29)
Hydrant Flushings	17	6.8-8.5 (7.8)	2.1-2.8 (2.4)	0.2-1.4 (0.78)	0.0 -1.4 (0.22)	0.4-1.4 (1.0)	21-40 (32)
Water System III							
Raw Water	4	6.4-7.4 (6.9)	7.6-8.2 (7.9)	0	2.83-3.06 (2.92)	0.3-0.4 (0.35)	48-60 (54)
Consumers' Taps	10	6.5-8.5 (7.4)	2.1-2.8 (2.4)	0.5-2.0 (1.1)	0.02-0.1 (0.05)	0-0.1 ([0.1)	46-55 (51)
Hydrant Flushings	31	6.2-8.4 (7.4)	1.8-4.8 (3.0)	0.1-1.3 (0.5)	0.1 -1.2 (0.44)	0-0.5 (0.13)	42-57 (49)

Column a - Range of water quality analysis for samples tested
Column b - Average value of water quality analysis for samples tested
Water System I - Deep wells
Water System II - Surface impoundment
Water System III - Shallow wells

TABLE II. PERCENT FREQUENCY OF THE MICROBIAL POPULATIONS OBSERVED IN WATER SAMPLES FROM THREE WATER SUPPLY SYSTEMS

SAMPLE SOURCE	NUMBER OF SAMPLES TESTED	SULFATE-REDUCING ORGANISMS	SULFUR-OXIDIZING ORGANISMS	IRON-PRECIPITATING ORGANISMS	NITROGEN-FIXING ORGANISMS	NITRATE-REDUCING ORGANISMS	NITRIFYING ORGANISMS	APPENDAGE-PRODUCING ORGANISMS	ACTINO-MYCETES
Water System I									
Raw Water	7	42.8	42.8	42.8	57.1	NM	0	57.1	28.5
Consumers' Taps	15	20.0	66.7	53.3	46.7	42.8	0	40	26.7
Hydrant Flushings	9	100	100	100	66.7	50	33.3	88.9	22.2
Water System II									
Raw Water	4	100	25	100	25	50	0	75	75
Consumers' Taps	15	6.7	46.7	38.5	46.7	50	9.1	33.3	33.3
Hydrant Flushings	17	82.3	64.7	87.5	66.7	35.7	70.6	29.4	
Water System III									
Raw Water	4	50	100	75	100	100	66.7	0	50
Consumers' Taps	10	0	50	50	60	70	10	20	50
Hydrant Flushings	31	74.2	74.2	83.9	64.5	61.3	16.1	40	41.9

significantly different from those taken at consumers' taps. In general, hydrant flushing samples exhibited higher concentrations of iron and dissolved sulfide ions and lower concentrations of chlorine residuals. Physicochemical variables did not show any significant covariance between any two variables when regression analyses were performed on pooled data from all samples.

Table II indicates percent frequency of the eight major groups of microbial populations observed in both raw and treated water samples from these water systems. They include dissimilatory sulfate-reducing organisms (e.g., Desulfovibrio), sulfur-oxidizing organisms (e.g., Thiobacillus), iron-precipitating organisms (non-specific iron bacteria), nitrogen-fixing organisms (e.g., Azotobacter), nitrate-reducing organisms, nitrifying bacteria (e.g., Nitrosomonas and Nitrobacter), appendage/stalk-producing organisms (e.g., Hyphomicrobium and Caulobacter) and Actinomycetes (e.g., Nocardia). In general, treated water samples taken at hydrant flushings exhibited higher percent frequencies of these groups of microorganisms than those samples taken at consumers' taps. Since hydrant flushing samples often contained sediments from water distribution mains, it was postulated that more organisms might have been associated with pipe sediments than with the flowing water. Except for the nitrifying bacteria most of these organisms were also observed in their raw water samples.

In 1978, Ainsworth (28) reported his studies on the chemistry and microbiology of the conveyed water and deposits of fourteen United Kingdom distribution systems. He found a positive correlation between the bacterial numbers and the decrease in organic carbon throughout the distribution systems. Ainsworth also observed that water samples with plate counts greater than 10^3 per ml were frequently associated with the deterioration in water quality.

Allen et al. (29) conducted a survey of water main encrustations obtained from several water systems in the United States. With the scanning electron microscopy technique they have demonstrated that the microorganisms were attached predominantly at the surface or near the surface of the most tubercles examined.

Microorganisms that can potentially proliferate in potable water are limited only by the availability of assimilable substrates and growth conditions. Recently, Lee (27) examined the potential growth of tap water organisms by recirculating the tap water in a simulated pipe loop system. Tap water which contained an average value of 0.5 mg/l total organic carbon was chlorinated and pssed through a 0.45 um filter cartridge. She observed that within a few days the bacterial density of recirculated water reached 10^4 to 10^5 colony forming units (cfu) per ml even though the influent tap water contained less than 10 cfu per ml. Victoreen (30) reported consistently higher plate counts when he used an eight-fold diluted standard methods plate count medium supplemented with iron.

To date only a limited effort is being directed toward characterizing the microorganisms observed in treated water. The major obstacle is inadequate isolation method for culturing the majority of dormant organisms observed in drinking water. When Parson et al. (31) examined the effluent from a pilot granular activated carbon system to identify the observed bacteria, they noted that many organisms did not survive during subculturing processes. Lee (27) also observed similar findings of decreased viabilities when bacteria from tap water samples were subcultured on non-selective media such as trypticase soy agar, nutrient agar, tryptone glucose agar, etc. Decreasing the nutrient concentrations had resulted in better growth response, i.e., larger colonies and longer survival in growth media. Thus, the current standard plate count analysis many bacteria would go undetected.

In spite of the lack of any standardized identification procedure, several studies have been reported on the microorganisms present in plate count media with their morphological and biochemical characteristics. Olivieri and Snead (32) examined a number of samples from various locations in the distribution systems of Baltimore and Frederick, Maryland. Their study was limited to microorganisms which formed colonies in standard plate count procedures. Despite the variability in the groups of microorganisms between sampling stations and between samples taken on different dates, the overwhelming majority of the greater than 6000 isolates collected were gram negative, non-saccharolytic rod shaped bacteria. A large percentage of the organisms formed pigmented colonies. The biochemical characteristics of the aerobic and facultative anaerobic group were consistent with the members of the family Pseudomonadaceae and the genera Flavobacterium, Alcaligenes, Acinetobacter and Moraxella. These same genera of microorganisms were also reported in other water distribution systems (27,29,33,34,35).

Geldreich (personal communication) has stated that investigators from many parts of the country are reporting the same predominant types of bacteria in water distribution systems. Relative numbers vary, however, as do the presence of non-dominant types of organisms.

Le Chevallier, et al. (33) also investigated the bacterial population in water samples collected from the finished drinking water supply of an Oregon coastal community. He found the number of standard plate count bacteria ranged from less than 0.02 to over 10^4 bacteria per ml, whereas total coliform numbers ranged from 4 to 440 per 100 mls. They have also enumerated nearly 700 isolates from colonies growing on membrane filters cultured on M-SPC medium. It was found that the actinomycete group (10.7%) comprised the largest portion of the M-SPC population and Aeromonas spp. (9.5%), the second largest group in

chlorinated drinking water samples examined. Acinetobacter spp. was the most commonly isolated microorganisms and was present in almost every sample. Of the 347 bacteria that were identified 30% of bacteria were those that may be considered opportunistic pathogens. They include, Pseudomonas maltophila, Pseudomonas fluorescens, Pseudomonas cepacia, Pseudomonas mallei, Acinetobacter spp., Klebsiella pneumoniae, Staphylococcus aureus, Moraxella spp., and Serratia liquefaciens. The following bacteria were considered antagonistic to coliforms: Pseudomonas, Micrococcus Flavobacterium, Bacillus and Actinomycetes. These comprised 35% of the M-SPC bacteria identified. Gram positive bacteria comprised nearly 36% of the M-SPC bacteria isolated from chlorinated drinking waters. These authors noted that gram positive bacteria in the chlorinated distribution water samples comprised nearly three times the number found in raw water samples. It may be that chlorination selected for gram positive bacteria.

Van der Kooj (36) examined 144 samples of tap water and found that Bacillus accounted for 18% of 752 isolates, coagulase negative, catalase positive staphylococci 8%, Aeromonas, 6% and the rest Gram negative rods. When the growth medium was tenfold-diluted and incubation period was extended up to three weeks, the colony counts at 22°C yielded 10 to 1000 times higher counts than the standardized procedure of 37°C at 48 hours incubation period.

Although the direct count procedure for the enumeration of bacteria has been used in aquatic and soil microbiology fields for many years (37), it has not been used in the analysis of microbiological quality of potable waters. In our preliminary studies of tap waters using epifluorescent direct count method with acridine orange fluorochrome it was shown that up to two orders of magnitude higher counts of bacteria could be detected as compared to that of standard plate count method. Similarly, Herson and Baker (38) reported up to three orders of magnitude difference in bacterial counts in their study of well and

distribution water samples. Their direct count method combined acridine orange staining with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride reduction. The latter technique measures the number of actively respiring bacteria in a sample of total bacteria.

C. Research Procedures

1.1 Water Supply Systems

The water system I is a large metropolitan city in the southeastern part of the United States. Raw water is transported from a large river into two holding reservoirs located near the treatment plant. Water treatment processes consist of rapid mix with lime and alum additions, presedimentation basin with powered activated carbon addition, coagulation/flocculation, sedimentation, duel media filtration and disinfection. Polyphosphate is added to the system from time to time to retard the corrosion of water mains.

The water system II is a smaller city system whose water source is a surface impoundment. This system is located in the northeastern part of the United States, and its water treatment processes consist of rapid mix, coagulation/flocculation, sedimentation, filtration and disinfection. The treated water is transported to an open storage basin and then pumped to its distribution system.

1.2 Sampling Techniques

Water samples were collected either at consumers' taps or at fire hydrant valves. The openings of water faucets and hydrant valves were washed with 70 percent ethyl alcohol and flame sterilized. Sufficient flushing times, (i.e., up to 20 minutes for tap sampling and 40 minutes for hydrant sampling) were employed in order to collect only the water samples from the distribution mains. Sterile 0.2% sodium thiosulfate solution was used as the dechlorinating agent.

1.3 Sampling Locations

The major portion of this study was conducted with water samples taken from the water supply system I. In particular, two (20 inch and 8 inch diameter) cement lined galvanized steel mains were selected because they contained fewer service connections than other mains. Sites 1, 2, 3 and 4 were taken from the 20 inch main, and they are located about 0.16 km, 2.7 km, 8.2 km and 11.4 km from the treatment plant, respectively. Sites 5, 6 and 7 were from the 8 inch main, and they are located about 4 km, 6.7 km and 8 km from the treatment plant, respectively. Site 8 is located at Georgia Tech Civil Engineering Laboratory, about 3 km from the water treatment plant.

For the direct microscopic enumeration of bacteria both water samples from water systems I and II were used.

1.4 Experimental Procedures

The procedures used during these investigations were those recommended in Standard Methods (39) sections 900 through 911, and those specified in the following discussion.

After conducting a comprehensive literature review of previous investigations on aquatic and soil microorganisms, the following list of selective enrichment culture media were chosen for this study. They include media to selectively enrich for the aerobic heterotrophic bacteria, anaerobic heterotrophic bacteria, iron-precipitating bacteria, sulfate-reducing bacteria, sulfur-oxidizing bacteria, nitrogen-fixing bacteria, nitrifying bacteria, denitrifying bacteria, appendage/stalk-producing aquatic bacteria, actinomycetes, yeasts and molds.

In order to identify the predominant group of microorganisms in potable waters previously known enrichment culture media were modified with respect to their chemical constituents and concentrations. (40-48) Table III lists the

TABLE III. LISTS OF SELECTIVE ENRICHMENT CULTURE MEDIA, PREDOMINANT MICROBIAL POPULATIONS AND ENRICHMENT MEDIA COMPOSITIONS

MEDIA[b]	ENRICHED GROUPS OF MICROORGANISMS	CHEMICAL CONSTITUENTS OF MEDIA (per 1 liter of water)	
1. thioglycollate medium	anaerobic heterotrophs		
2. modified Winogradsky's iron precipitating medium	iron-precipitating heterotrophs	ferric ammonium citrate	5.0g
		NH_4NO_3	0.5g
		$MgSO_4 \cdot 7H_2O$	0.5g
		K_2HPO_4	0.5g
		$CaCl_2 \cdot 6H_2O$	0.2g
		pH adjusted to 6.8 with KOH	
		trace element	1 ml
3. Stoke's medium	filamentous iron bacteria (S. natans)	glucose	1.0g
		peptone	1.0g
		$FeCl_3 \cdot 6H_2O$	0.1g
		$MgSO_4 \cdot 7H_2O$	0.2g
		NaCl	0.1g
		$CaCl_2 \cdot 2H_2O$	0.025g
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		trace element	1 ml
		pH 7.0	
4. reinforced Baar's medium	sulfate-reducing bacteria; anaerobic bacteria	sodium lactate	3.5 ml
		yeast extract	1.0g
		NH_4Cl	1.0g
		$MgSO_4 \cdot 7H_2O$	2.0g
		$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.5 to 0.8g
		sodium thioglycollate	0.6g
		ascorbic acid	0.1g
		K_2HPO_4	0.5 to 1.0g
		trace element	1 ml
		pH 7.0	

TABLE III (Continued)

5. thiosulfate mineral medium	sulfur-oxidizing/precipitating organisms	sodium thiosulfate	10g
		NH_4Cl	0.5g
		$MgSO_4 \cdot 7H_2O$	0.2g
		NaCl	0.1g
		$FeCl_3$	1 mg/l
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		yeast extract	0.05g
		trace element	1 ml
		pH 6.9	
6. nitrogen-free medium	nitrogen-fixing heterotrophs	mannitol	5.0g
		$MgSO_4 \cdot 7H_2O$	0.2g
		$NaMoO_4 \cdot 2H_2O$	0.05g
		NaCl	0.1g
		$CaCO_3$	0.5g
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		trace element	1 ml
		pH 7.2	
7. modified Winogradsky's mineral medium without carbon source	autotropic nitrifying bacteria	$(NH_4)_2SO_4$	1.0g
		$MgSO_4 \cdot 7H_2O$	0.5g
		NaCl	0.1g
		$CaCO_3$	1.8g
		$CaCl_2 \cdot 2H_2O$	0.025g
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		trace element	1 ml
		pH 8.0	

TABLE III (Continued)

8. dilute organic mineral medium	stalk-producing heterotrophs (e.g., Caulobacter and Hyphomicrobium sp.	peptone	0.1g
		$MgSO_4 \cdot 7H_2O$	0.2g
		$(NH_4)_2 \cdot 2H_2O$	0.5g
		$CaCl_2 \cdot 2H_2O$	0.025g
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		NaCl	0.1g
		trace element	1 ml
		pH 7.2	
9. basal mineral medium with nitrate	denitrifying heterotrophs	glucose	1.5g
		$NaNO_3$	1.5g
		$MgSO_4 \cdot 7H_2O$	0.2g
		NaCl	0.1g
		$CaCl_2 \cdot 2HO$	0.025g
		K_2HPO_4	1.1g
		KH_2PO_4	0.7g
		trace element	1 ml
		pH 7.0	
10. AGS - actinomycetes medium	actinomycetes	arginine	1.0g
		glycerol	12.5g
		$MgSO_4 \cdot 7H_2)$	0.5g
		K_2HPO_4	1.0g
		NaCl	1.0g
		$Fe(SO_4)_3 \cdot 6H_2O$	0.01g
		$CuSO_4 \cdot 5H_2O$	0.01g
		$ZnSO_4 \cdot 7H_2O$	0.001g
		$MnSO_4 \cdot 4H_2O$	0.001g
		pH 6.9	

TABLE III (Continued)

11. Czapek's medium	actinomycetes	glucose	15g
		glycerol	15 ml
		$MgSO_4 \cdot 7H_2O$	0.5g
		KCl	0.5g
		$FeSO_4 \cdot 7H_2O$	0.01g
		K_2HPO_4	0.1g
		pH 7.0	
12. yeast extract glucose medium	yeasts and molds	glucose	10g
		peptone	3.0g
		yeast extract	5.0g
		pH adjusted to 3.8 after sterilization	

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[a]trace element mixture

EDTA	5.0g
$ZnSO_4 \cdot 7H_2O$	2.2g
$MnSO_4 \cdot 4H_2O$	0.57g
$FeSO_4 \cdot 7H_2O$	0.5g
$CoCl_2 \cdot 6H_2O$	0.161g
$CuSO_4 \cdot 5H_2O$	0.157g
$NaMoO_4 \cdot 2H_2O$	0.151g
600 mls H_2O, heat the solution to dissolve the elements, adjust pH to 6.0 with 40% KOH, add water to 1 liter volume.	

[b]For solid medium, 15.0g agar was added per 1 liter of liquid medium.

compositions of the selective enrichment media used for this study. All media were sterilized at 121°C for 15 minutes and then distributed into sterile tubes. For anaerobic and microaerophilic organisms screw-capped tubes with inoculum were completely filled with anaerobic culture media. Duplicate water samples ranging from 5 mls to 50 mls of inocula were aseptically inoculated into equal volume of media. Inoculated tubes or flasks were then incubated at 25°C for periods up to three weeks to ensure adequate incubation time for the slow growing organisms. The enumeration of predominant microorganisms in potable water was determined by pour plate techinques using the solid form of various selective enrichment culture media. The cultivation of anaerobic organisms was conducted in an anaerobic chamber (Forma Scientific Model 1024) with 85% N_2, 10% CO_2 and 5% H_2.

In order to develop an improved procedure for the enumeration of viable heterotrophic bacteria from drinking waters, various alternative methods were investigated. Initially, the current standard plate count procedure was modified to include longer incubation times and lower incubation temperatures. Alternative new media were also evaluated. They include R2A, diluted standard method agar, m-standard plate count media and basal mineral media. The formulations of these media are shown in Table IV. The incubation period ranging from 2 to 14 days were examined, and three incubation temperatures 20°, 25° and 35°C, were evaluated in order to determine the optimum culturing condition. When fewer than 30 colony forming units (CFUs) were observed per ml of water sample, both larger volume of inoculum and larger petri dishes (150 mm diameter) were employed. In addition, both spread plating and membrane filter techniques were examined in order to compare these two procedures to that of the current standard method.

TABLE IV. PLATE COUNT MEDIA AND THEIR COMPOSITIONS

MEDIA	CHEMICAL CONSTITUENTS OF MEDIA (per 1 liter of water)	
1. diluted tryptone glucose medium	trypticase peptone	0.5g
	yeast extract	0.25g
	glucose	0.1g
	agar	15.0g
2. R2A medium	yeast extract	0.5g
	proteose peptone #3	0.5g
	casamino acids	0.5g
	glucose	0.5g
	soluble starch	0.5g
	K_2HPO_4	0.3g
	$MgSO_4 \cdot 7H_2O$	0.05g
	sodium pyruvate	0.3g
	agar	15.0g
3. M-standard plate count medium	peptone	20g
	gelatin	25g
	glycerol	10g
	agar	15g
4. Basal mineral medium	carbon source	1%
	NH_4Cl	0.5g
	$MgSO_4 \cdot 7H_2O$	0.2g
	K_2HPO_4	1.1g
	KH_2PO_4	0.7g
	$CaCl_2 \cdot 2H_2O$	0.025g
	trace element	1 ml
	pH 7.0	

Preliminary identification of microorganisms observed in selective enrichment culture media, m-Endo media and enumeration plate count media include colonial morphology, direct microscopic examinations, stain reactions and biochemical reactions such as oxidase test, catalase test, urease test, hydrogen sulfide production test, methyl red test, Voges-Proskaauer test, starch hydrolysis, nitrate reduction test and carbohydrate oxidation-fermentation tests. The Analytab product, API/20E, a preset battery of 20 microtubes for the performance of 22 biochemical tests for identification of Enterobacteriacea and other Gram negative bacteria, and Corning's non-fermenter system, a commercial identification system for Gram negative bacteria, were utilized in order to identify the isolates subcultured from various culture media.

To determine the total bacterial density of potable waters, a modification of epifluorescent direct count method (49-53) was used. Dechlorinated water samples were fixed with filtered formalin [1:1(v/v)] formaldehyde (37%) and McIlvaines' buffer (pH 7) at the final concentration of 1.85%. Other concentrations of fixatives ranging from 0.185% to 3.7% were also examined. Nucleopore filter (25 mm diameter, 0.2 um pore size) was prestained with an Irgalan black solution (0.2 g per 100 mls of water which contains 2 mls of glacial acetic acid) for better visibility of fluorescent bacteria. Before replacing a nucleopore membrane filter a damp backing filter (25 mm diameter, 0.45 um pore size cellulose acetate filter) was placed on top of the filter receptacle in order to obtain an even distribution of bacteria on the filter surface.

Two fluorchrome stains, acridine orange (AO) and 4'6-diaminido-2-phenylindole (DAPI) were examined. Stock solutions of AO (500 ug/ml) and DAPI (200 ug/ml) were prepared with freshly distilled water which has been membrane filtered with a 0.2 um pore size membrane filter and autoclaved. Both stock

solutions were stored at 4°C. Before use, the stock solutions were diluted to their respective final stain concentrations with filter sterilized McIlvaine's buffer (pH 7). The optimum staining condition was selected on the basis of the maximum number of stable fluorescent cells observed on filters. Both stain concentrations and contact times were varied to determine the optimum staining procedure. The fluorochrome concentrations evaluated range from 10 ug/ml to 200 ug/ml for acridine orange stain and 0.05 ug/ml to 20 ug/ml for DAPI stain. The contact times tested were between 2 min. to 20 min for both stains.

For the direct microscopic enumeration of metabolically active cells methodologies of DAPI and INT techniques were combined. Briefly, the technique was as follows. Samples were incubated in 0.02 percent (final concentration) INT solution for 40 min. in the dark environment and then fixed with formalin (1.85% final concentration). After fixation, the sample was filtered through a prestained nucleopore filter. Three ml of DAPI stain was added to the filter with collected cells and allowed to stand for 5 min. The stained cells were transferred to a gelatin matrix by using the procedure of gel stripping, which has been described by Herson and Baker. (38) Briefly, this procedure involves placing the stained filter face down on a cover slip or slide that has been precoated with a warm solution of a mixture of gelatin (5 percent) and chrom alum (0.05 percent). The gelatin is allowed to dry for several hours. The dried filter is dipped in 1% glycerol solution in order to facilitate its removal from the slide. The cells were embedded in the gelatin mixture. Finally, the slide was redipped in the gelatin solution to cover the embedded cells and to prevent the solubilization of the INT-formazan granules by immersion oil. The stained preparations were examined under both epifluorescent and bright-field microscopes.

The Olympus research microscope (Model vanalux) which is equipped with both epifluorescence and bright-field illumination and objective was used for direct counting of fluorescent cells. Metabolically active cells exhibited bright red INT formazan granules within bacterial cells.

D. Results and Discussion

The principal objective of this research was to examine both qualitative and quantitative compositions of microorganisms present in drinking waters. The initial phase of this study was focused on the characterization of the major groups of microbial populations in drinking water by means of enrichment culture techniques. Since one rarely encounters a pure culture of bacteria in natural habitats, the use of enrichment culture techniques can be applicable in the qualitative determination of physiologically different groups of microbial populations in water. Based on our previous investigations of drinking water systems in Missouri (27), twelve selective enrichment culture media were chosen for this study. They include those organisms which are directly or indirectly involved in transformation of elements, such as carbon, nitrogen, sulfur and iron in water. Between July and December 1980 forty-two samples from seven sampling locations of Atlanta water distribution system were examined. The positive growth of each group of microbial population was determined by means of microscopic observations and by the resultant biochemical changes of the media. In particular, both non-specific iron precipitating and filamentous iron bacteria produced ferric hydroxide precipitate in the culture media. Dissimilatory sulfate-reducing bacteria produced hydrogen-sulfide which then reacted with the available ferrous ions in the medium and formed black iron sulfide precipitate. Sulfur oxidizing organisms produced sulfur precipitates in the media.

Microbiological analysis of forty-two water samples taken at consumers' taps indicated that about 70% of samples contained iron bacteria, nitogren-fixing bacteria and appendage/stalk-producing bacteria. Actinomycetes and sulfur-oxidizing groups were seen in about 45% of the samples, whereas anaerobic sulfate-reducing bacteria and nitrate-reducing bacteria were observed in only about one-third of the samples tested. Less than 5% of the samples contained nitrifying bacteria. Microbiological analysis of water samples taken at hydrant flushings exhibited generally higher percent frequencies of microbial groups than those samples taken at consumers' taps. In particular, more than 50% of the water samples obtained at hydrant flushings contained both sulfate-reducing bacteria and nitrate-reducing bacteria. Our earlier studies of Missouri water systems also yielded higher percent frequencies of various microbial groups in hydrant flushing samples. Since microorganisms can form micro-colonies on the surface of water distribution pipes, one would expect higher density of organisms in hydrant flushing samples which contained both pipe sediments and water.

For enumeration of various microbial populations in drinking water 1%-1.5% agar was added to various selective enrichment culture media as specified in Table III. Pour plate technique was employed in the enumeration of various microorganisms. Table V indicates the summary of the bacterial plate counts on six isolation media for five sets of water samples taken at seven sampling locations. Although it is not readily apparent from Table V, over 80% (81.5%) of all the plates examined contained less than 10 colony forming units per ml of water sample. In terms of the overall number of organisms in drinking water, the filamentous iron bacteria ranked the first. The next predominant group of organisms was actinomycetes. The nitrogen fixing bacteria ranked third in terms of their density in water. It is impossible to enumerate accurately all the

TABLE V. BACTERIAL PLATE COUNT ANALYSIS

SELECTIVE ENRICHMENT MEDIA	SAMPLE LOCATIONS													
	1		2		3		4		5		6		7	
	N	S.D.	N	S.D.	N	S.D.	N	S.D.	N	S.D.	N	S.D.	N	S.D.
Iron Precipitating Bacteria Medium	20	14	34	10	30	25	104	125	28	19	81	35	280	67
Filamentous Iron Bacteria Medium	38	26	84	73	145	125	224	200	117	66	112	34	656	358
Sulfur Oxidizing Bacteria Medium	19	5	50	44	14	14	79	51	83	88	109	126	162	141
Nitrogen-Fixing Bacteria Medium	49	23	57	53	140	86	208	115	73	37	94	20	386	134
Stalk/Appendage Producing Bacteria Medium	47	16	51	26	112	103	96	30	26	14	91	16	268	80
Actinomycetes Medium	102	106	139	133	55	32	283	271	16	15	80	9	473	425

N represents the arithmetic average number of organisms observed per 10 ml water sample for the total sampling period.

S.D. represents the overall standard deviation.

$$*S.D. = \sqrt{\frac{n \sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2}{N(n-1)}}$$

where X_i = the total number observed per sample
n = the total number of samples

bacteria present in water; however, this study indicated that selective enrichment culture techniques may be very useful and applicable for the determination of predominant microbial groups in water.

With respect to different sampling locations the highest number of bacteria was consistently observed with water samples taken from the smaller 8 inch water main at the farthest distance from the treatment plant (i.e., No. 7). It is particularly noteworthy that even though the sample location No. 4 is further from the water treatment plant than the sample site No. 7, the latter samples contained more bacteria per volume of water. Although overall bacterial densities of water samples were low, there was a general trend of increasing bacterial densities present in water samples which were taken at sampling locations further from the water treatment plant.

The identification of microorganisms is a difficult task, at best, and it is based predominantly on observed morphological and biochemical characteristics.

The bacterial diversity of drinking water samples was investigated by systematically examining the morphologically different colonies that grew on various isolation media. Descriptions of various isolates according to their colonial and cellular morphologies on various isolation media at various sampling locations and dates are indicated in Appendix A. Each isolate represents the cluster of species which exhibited a particular characteristic of metabolic response in its corresponding selective enrichment culture media.

The majority of isolates that grew on various selective enrichment culture media were pigmented. In particular, about two-thirds (67%) of the colonies that grew on iron-precipitating media were pigmented, and nearly half (48%) of the isolates observed in low organic mineral culture media were also pigmented. The relative occurrence of pigmented colonies on standard plate count and R2A media were compared, and data are presented in Table VI.

TABLE VI. PERCENT OF 14-DAY COLONIES THAT WERE PIGMENTED

SITE	DATE	MEDIA	AT 20°C INCUBATION			AT 25°C INCUBATION			AT 35°C INCUBATION		
			WHITE	YELLOW & ORANGE	PINK	WHITE	YELLOW & ORANGE	PINK	WHITE	YELLOW & ORANGE	PINK
1	8-19-80	SPC	96 % (25)	4 % (1)	0 % (0)	98 % (44)	0 % (0)	2 % (1)	90 % (36)	10 % (4)	0 % (0)
		R2A	94 % (44)	2 % (1)	4 % (2)	90 % (69)	9 % (7)	1 % (1)	---	---	---
2	8-11-80	SPC	Some	Most	Very few	---	---	---	Some	Most	Very few
		R2A	Some	Most	Very few	---	---	---	Some	Most	None
	8-19-80	SPC	66 % (45)	10 % (7)	24 % (16)	54 % (43)	26 % (21)	20 % (16)	69 % (70)	8 % (8)	23 % (23)
		R2A	40 % (93)	25 % (58)	35 % (83)	39 % (88)	18 % (42)	43 % (99)	56 % (58)	24 % (25)	20 % (20)
	11-13-80	SPC	94 % (136)	4 % (6)	2 % (3)	91% (152)	7 % (12)	2 % (4)	84 % (42)	12 % (6)	4 % (2)
		R2A	89 % (328)	2 % (6)	9 % (32)	89 % (494)	2 % (9)	9 % (49)	80 % (54)	15 % (10)	5 % (3)
	11-17-80	SPC	97 % (352)	2 % (6)	1 % (3)	97 % (528)	2 % (11)	1 % (4)	93 % (121)	6 % (8)	1 % (1)
		R2A	99 % (1526)	0.2 % (3)	1 % (18)	95 % (1615)	1 % (12)	4 % (62)	93 % (138)	6 % (9)	1 % (1)
4	8-19-80	SPC	94 % (67)	3 % (2)	3 % (2)	97 % (63)	3 % (2)	0 % (0)	97 % (35)	3 % (1)	0 % (0)
		R2A	85 % (88)	3 % (3)	12 % (13)	---	---	---	---	---	---
	11-6-80	SPC	95 % (261)	2 % (6)	3 % (7)	87 % (32)	8 % (3)	5 % (2)	93 % (28)	7 % (2)	0 % (0)
		R2A	68 % (138)	5 % (9)	27 % (55)	50 % (45)	6 % (5)	44 % (39)	86 % (36)	12 % (5)	2 % (1)
SUMS FOR SPC			94 % (886)	3 % (29)	3 % (31)	92 % (862)	5 % (49)	3 % (27)	86 % (332)	7 % (29)	7 % (26)
SUMS FOR R2A			89 % (2217)	3 % (80)	8 % (203)	88 % (2311)	3 % (75)	9 % (250)	79 % (286)	14 % (49)	7 % (25)

The analysis of data indicated that although occurrences of pigmented organisms on both standard plate count media and R2A media were significant, non-pigmented organisms occupied the major portion of the aerobic and facultative anaerobic organisms. This study also indicated that R2A medium supported the higher number of pigmented organisms than the standard plate count medium for all three incubation temperatures. Reasoner and Geldreich (54) studies occurrences of pigmented bacteria in water supplies. Their study revealed that R2A medium clearly gave the higher counts at 48 hours incubation, and supported high percentage of pigmented bacteria.

During the course of this study we have examined about 300 isolates that were randomly selected from various selective enrichment culture media, m-Endo medium, and various plate count media. Many isolates had lost their viability upon subculturing unto their respective isolation media. The majority of colonies did not appear on various enrichment culture media until 6 to 7 days incubation, and most colonies remained pinpoint size during the entire cultivation period of up to three weeks. In addition, when isolates were examined for their identification by colonial morphology, cellular morphology, staining reactions, biochemical reactions, etc., many more isolates were identified as the repeat cultures from the other water samples. Cellular morphologies and gram reactions of these isolates are 31.3% Gram positive rods, 6.7% Gram positive cocci, 55.9% Gram negative rods, 4.4% Gram negative cocci and 1.7% yeasts and molds. For their subsequent identification isolates were grouped according to their reactions to oxidase test, catalase test, growths on MacConkey medium, triple sugar iron medium and OF glucose fermentation/ oxidative reaction. When the Analytab product, API/20E and other non-fermenting Gram negative organism identification tests were utilized, the majority of isolates exhibited inert reactions to various biochemical tests within 48 hours.

Consequently, identification methods were not used. Taxonomic identification was limited to their genera level, and isolates were divided into various subgroups according to their cultural and biochemical reactions (Appendix B). Some of the isolates tentatively identified included Enterobacter agglomerans, Acinetobacter cloaca, Enterobacter sp. Aeromonas sp. Chromobacterium violaceum, Vibrio parahaemolyticus, Bacillus spp. Pseudomonas maltophilia, P. cepacia, Flavobacterium spp., Moraxella sp. Alcaligenes sp., Yersinia sp., Azotobacter sp., Derxia sp., saprophytic Mycobacterium spp., Streptomyces, Nocardia spp., Sarcina lutea, Enterococcus spp., and a variety of yeasts and molds.

When our earlier studies of various water systems indicated that the most predominant group of microbial populations in finished water was aerobic and facultative anaerobic heterotrophic bacteria, a considerable effort was directed toward developing an improved culturing technique for this group of organisms.

A comprehensive evaluation of various plate count methods was performed on 13 water samples collected from Atlanta water system. Figures 1 through 4 are graphical presentations of the data for colony counts versus incubation time for a few samples tested with various plate count media and incubation temperatures. Results of plate count analyses of four media (SPC, R2A, m-SPC and SPC/10), three incubation temperatures (20°C, 25°C and 35°C) and two incubation periods (7 and 14 days) are presented in Tables VII and VIII. Although the actual water volumes employed range from 7 mls to 30 mls per plate, in order to facilitate the comparison, all colony counts were presented in colony forming units (cfu) per 30 ml of water. Figure 5 indicates the efficiency of modified plate count techniques as measured by their relative ratios to SPC at 35°C. Each bar represents a ratio of the average cfu value of three samples using a specified media (SPC, R2A, m-SPC or SPC/10) at a specified incubation time (7 or 14 days) and temperature (20°C, 25°C or 35°C) to the

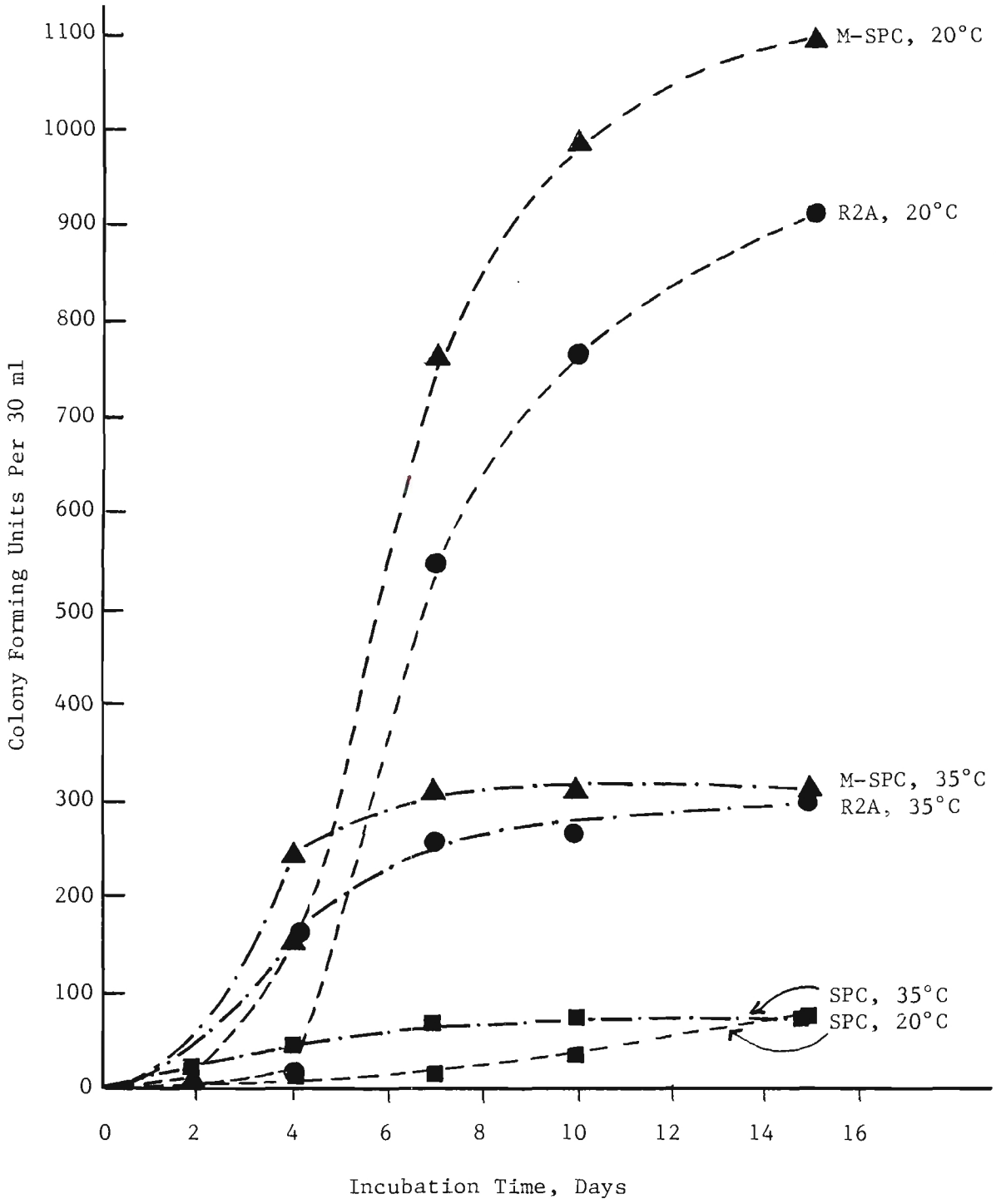

Figure 1. Colony Forming Units Per 30 ml Water Cultured on SPC, R2A and M-SPC Media at 20°C, 25°C and 35°C Incubation Temperatures vs. Incubation Times for Site No. 3, 8-11-80

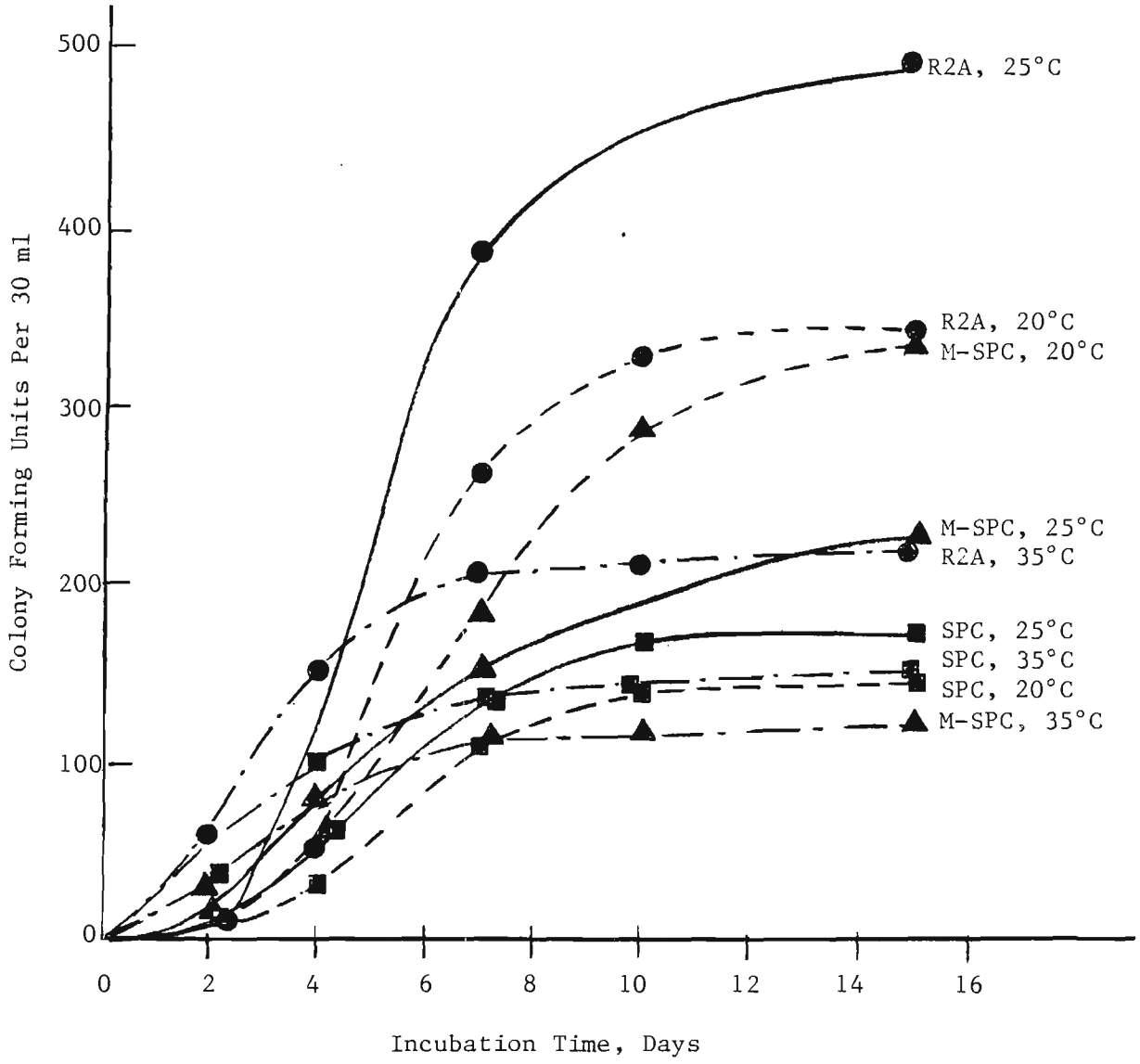

Figure 2. Colony Forming Units Per 30 ml Water Cultured on SPC, R2A and M-SPC Media at 20°C, 25°C and 35°C Incubation Temperatures vs. Incubation Times for Site No. 2, 8-19-80

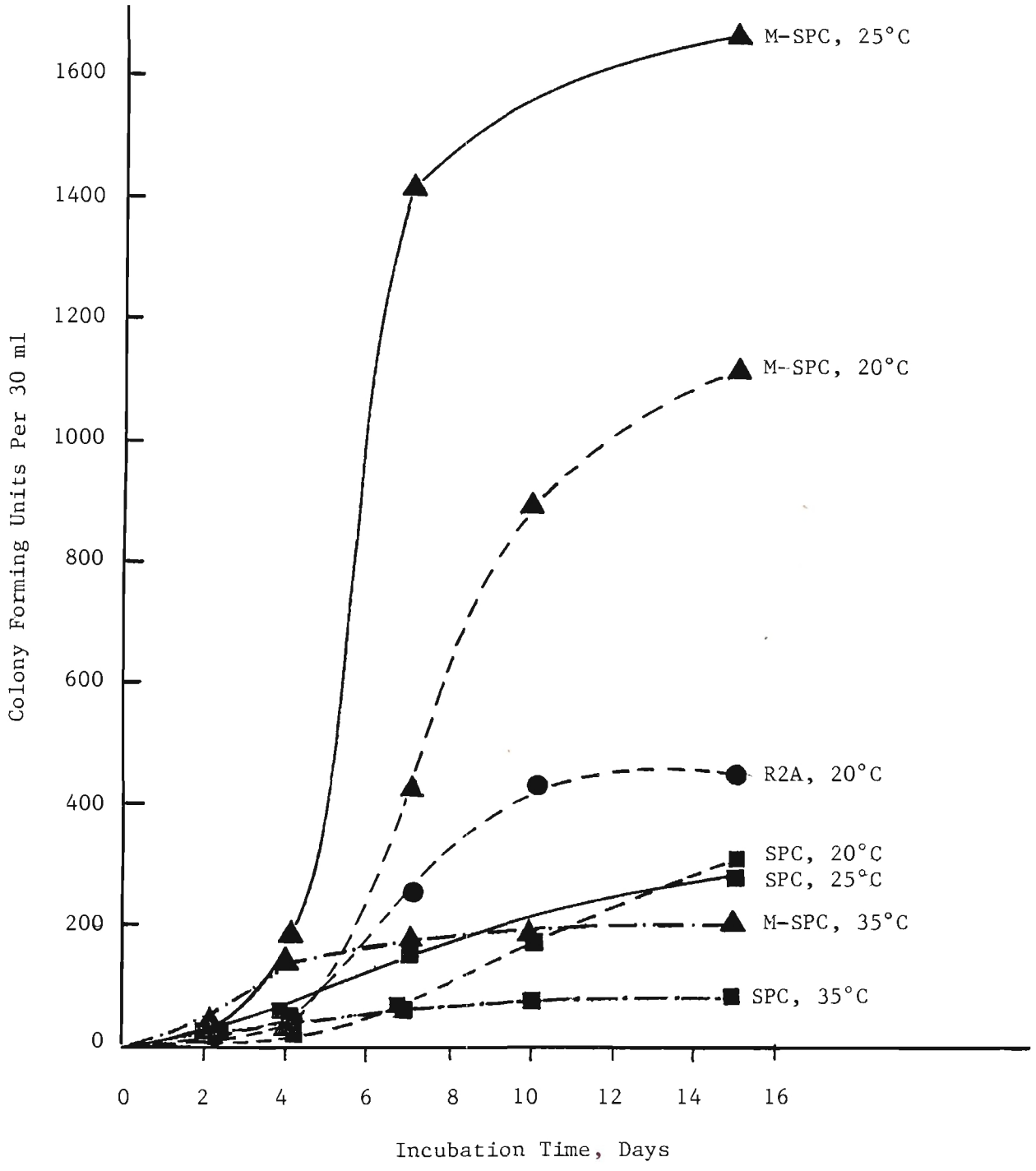

Figure 3. Colony Forming Units Per 30 ml Water Cultured on SPC, M-SPC and R2A Media at 20°C, 25°C and 35°C Incubation Temperatures vs. Incubation Times for Site No. 4, 8-19-80

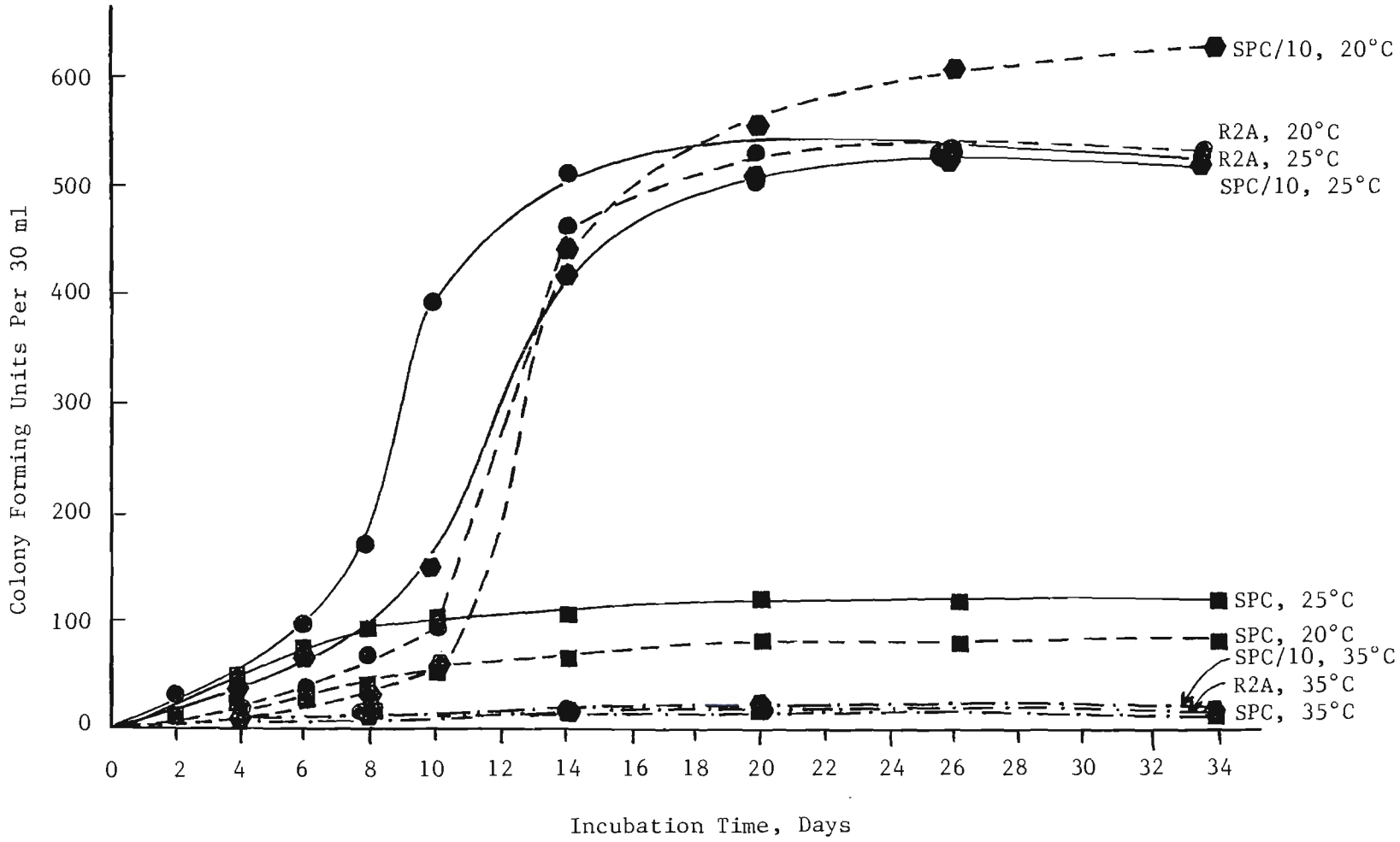

Figure 4. Colony Forming Units Per 30 ml Water Cultured on SPC, R2A, SPC/10 Media at 20°C, 25°C and 35°C Incubation Temperatures vs. Incubation Times for Site No. 2, 11-17-80

TABLE VII. SUMMARY OF 7-DAY COLONY COUNTS

Sample No.	Site No.	Sample Volume Per Plate (ml)	COLONIES/30 ML											
			20°C				25°C				35°C			
			SPC	R2A	M-SPC	SPC/10	SPC	R2A	M-SPC	SPC/10	SPC	R2A	M-SPC	SPC/10
1	1	7	<129	<129	<129	---	---	---	---	---	<129	<129	---	---
2	2	7	<129	267	374	---	---	---	---	---	164	191	176	---
3	3	7	<129	542	762	---	---	---	---	---	<129	256	306	---
4	4	7	---	---	---	---	---	---	---	---	<129	303	210	---
5	1	7	<129	<129	<129	---	<129	<129	<129	---	<129	---	<129	---
6	2	7	<129	263	183	---	133	390	147	---	132	206	<129	---
7	4	7	<129	253	423	---	149	---	1413	---	<129	---	176	---
8	5	7.5	222	261	261	---	240	357	256	---	140	312	265	---
9	4	20	<45	<45	<45	---	58	93	94	---	<45	<45	<45	---
10	2	30	<30	<30	---	<30	<30	38	---	<30	<30	<30	---	<30
11	6	30	138	186	---	158	193	283	---	286	<30	50	---	39
12	7	30	<30	<30	---	<30	<30	76	---	60	38	46	---	50
13	2	30	38	56	---	<30	78	95	---	75	<30	<30	---	<30

TABLE VIII. SUMMARY OF 14-DAY COLONY COUNTS

Sample No.	Site No.	Sample Volume Per Plate (ml)	COLONIES/30 ML											
			20°C				25°C				35°C			
			SPC	R2A	M-SPC	SPC/10	SPC	R2A	M-SPC	SPC/10	SPC	R2A	M-SPC	SPC/10
1	1	7	<129	<129	<129	---	---	---	---	---	<129	<129	---	---
2	2	7	140	328	461	---	---	---	---	---	164	192	197	---
3	3	7	<129	895	1077	---	---	---	---	---	<129	294	309	---
4	4	7	---	---	---	---	---	---	---	---	<129	329	230	---
5	1	7	<129	<129	<129	---	<129	<129	<129	---	<129	---	<129	---
6	2	7	146	341	330	---	171	487	222	---	146	217	<129	---
7	4	7	279	446	1098	---	266	---	1647	---	<129	---	198	---
8	5	7.5	241	327	300	---	246	369	258	---	138	318	270	---
9	4	20	100	441	117	---	70	177	225	---	<45	<45	<45	---
10	2	30	33	66	---	44	33	124	---	81	<30	<30	---	<30
11	6	30	231	345	---	338	234	438	---	420	<30	60	---	51
12	7	30	41	95	---	97	59	107	---	107	42	49	---	55
13	2	30	66	459	---	105	501	---	410	---	<30	<30	---	<30

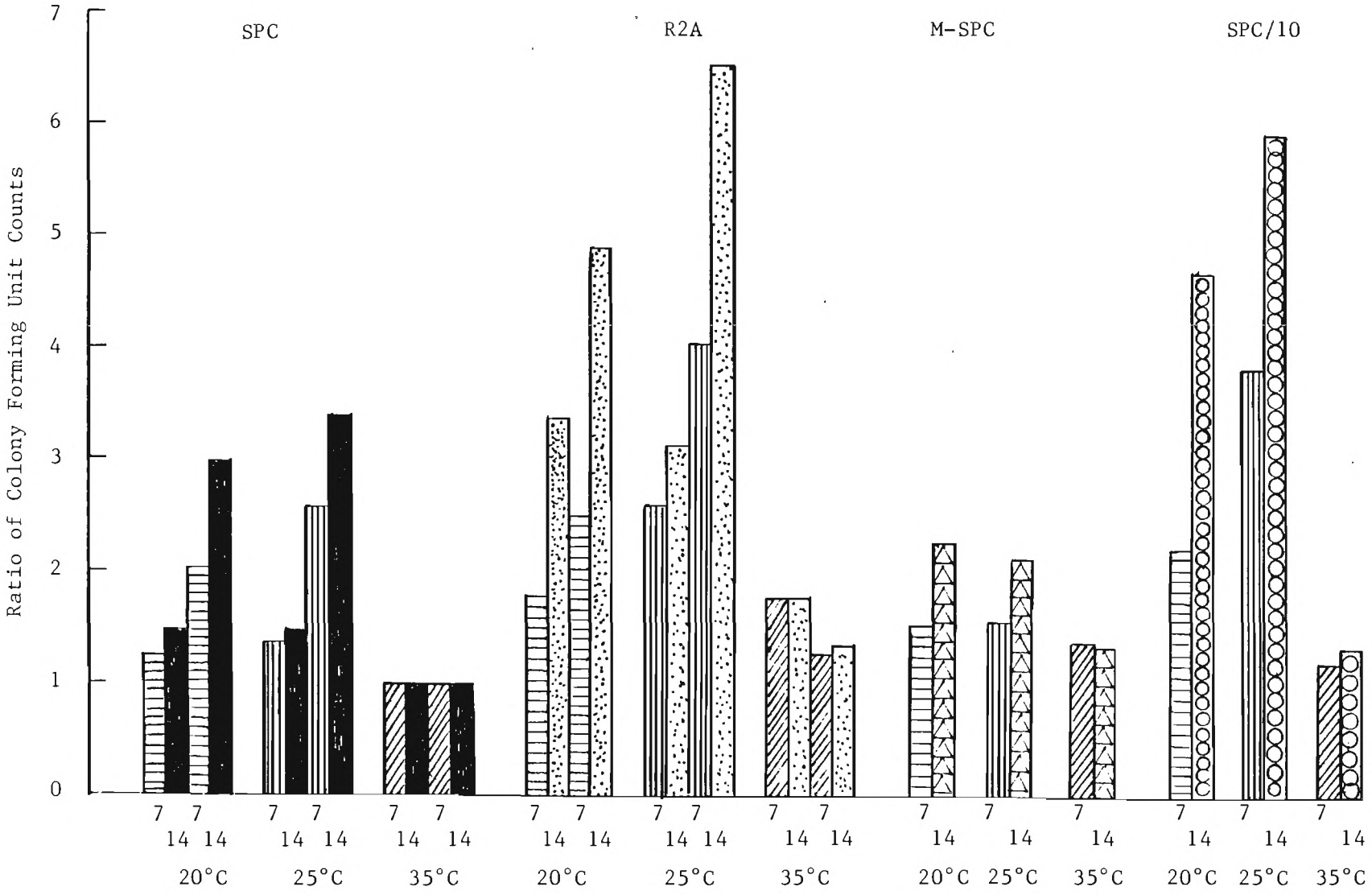

Figure 5. Ratios of Colony Forming Unit Counts Observed in SPC, R2A, M-SPC and SPC/10 Plates at 20°C, 25°C and 35°C Compared to CFU in SPC at 35°C

average cfu value of the same sample using SPC medium at 35°C. Analysis of data indicated that samples inoculated on R2A medium incubated at 25°C gave the highest ratio. Over six fold increase in colony counts was obtained when enumeration of aerobic heterotrophs was performed on R2A medium at 25°C with 14 days incubation period.

A ten-fold dilution of SPC medium at 25°C yielded the next high ratio (5.95) of colony counts, followed by ratios of 4.90 and 4.70 for R2A and SPC/10 at 20°C, respectively.

There is increasing agreement that it would be desirable to have a better indicator of bacterial quality than the coliform test because high numbers of bacteria may indicate the presence of nonfecal opportunistic pathogens, and high bacterial counts may indicate potential interference with detection of coliform bacteria. In addition, high concentrations of bacteria in drinking water may attribute to increased possibilities for taste, odor and discoloration problems in the distribution system. Since the current standard plate count analysis yielded the lower cfu than the other alternative technique, this may mean that a large proportion of bacteria that may be present in drinking water would be missed and undetected by the current method. Recent studies (55) indicated conventional physical, chemical and bacteriological qualities did not show any correlation to water quality problems that originate in distribution systems. It may be that the use of improved plate count techniques that yield higher bacterial densities and diversities may correlate better than the standard plate count with respect to the assessment of water treatment processes and water quality problems in distribution systems.

Quantitative measurements of total bacterial cells were determined by using a modified epifluorescent direct count method. Although the epifluorescent direct count procedure has been used for the enumeration of bacteria in a

variety of aquatic systems, its application in potable water system has not been explored in general. Initial studies were directed toward developing the optimum staining procedure for the maximum recovery of total bacteria in potable waters. Currently, there is no standard available against which the efficiency of the maximum recovery of total bacteria can be measured in potable waters; therefore, an operational definition of the optimum staining condition was given to a procedure at which the stained filter exhibited the maximum recovery of stable fluorescent cells. Results of the total number of fluorescent cells observed at various concentrations of two fluorochromes, acridine orange and DAPI, are shown in Figures 6 and 7, respectively. The maximum number of stable fluorescent cells was observed at 10 ug/ml acridine orange concentration at 5 min. contact time and 5 ug/ml DAPI final concentration at 10 min. contact time. For the analysis of potable water the concentration of acridine orange required for the maximum recovery of total bacteria was compatible to that of other aquatic systems; however the required DAPI concentration was significantly higher for potable water samples than those from other aquatic systems. This discrepancy may have been due to the presence of polyphosphate in potable waters. Though DAPI is a highly sensitive DNA stain, it can also react with polyphosphate producing yellow fluorescent DAPI-polyphosphate complex.

Results of total and metabolically active bacteria observed in 14 water samples from the water system I are shown in Table IX. When two fluorochromes, acridine orange and DAPI, were employed on the same water sample to compare their efficiency to stain bacterial cells in water, acridine orange staining exhibited the higher number of fluorescent cells than the DAPI staining. (Fig. 8) Acridine orange fluorescent cell counts ranged from 2082 to 6918 per ml of water, while DAPI stained cell counts ranged from 806 to 3446 per ml of water. This discrepancy is difficult to explain, since there is no standard available

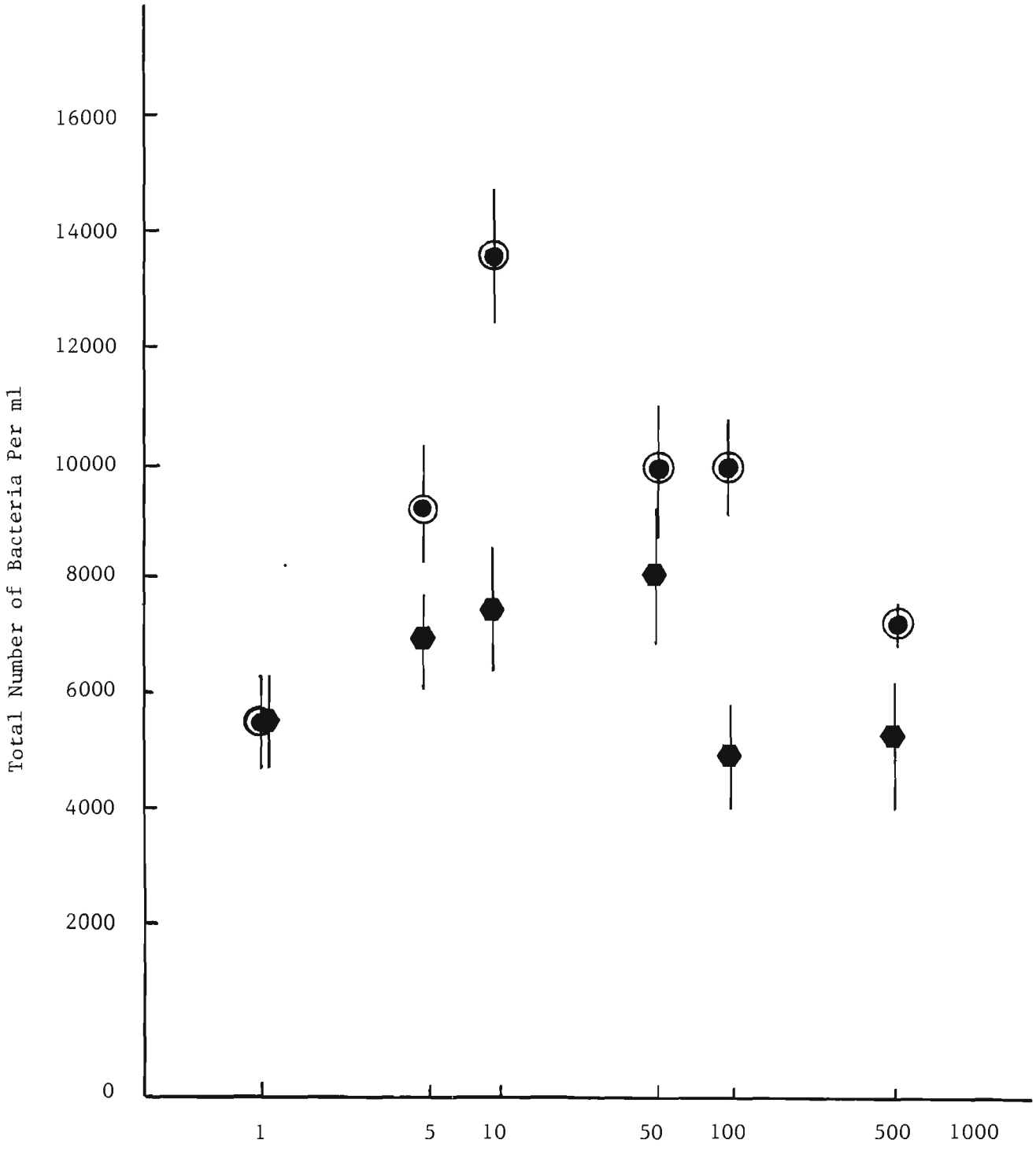

Figure 6. Epifluorescent Direct Count of Total Bacteria vs. Acridine Orange Concentration at 2 Min. Contact Time (-●-) and at 5 Min. Contact Time (-◉-)

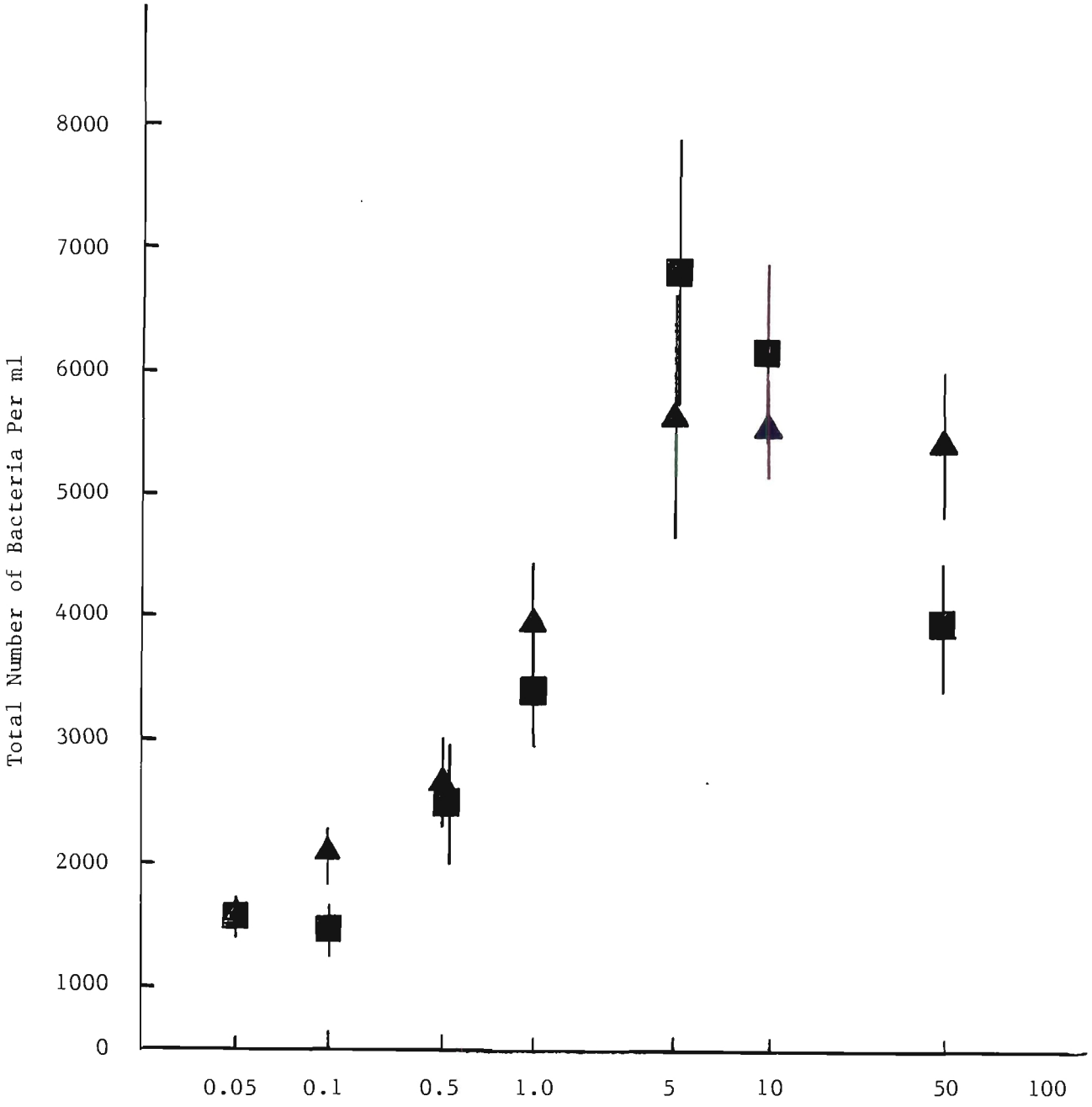

Figure 7. Epifluorescent Direct Count of Total Bacteria vs. DAPI Concentrations at 10 Min. Contact Time (▲) and at 30 Min. Contact Time (■)

TABLE IX. RESULTS OF EPIFLUORESCENT DIRECT COUNT (TOTAL BACTERIA AND METABOLICALLY ACTIVE BACTERIA (i.e., INT POSITIVE BACTERIA), STANDARD PLATE COUNT AND CHLORINE RESIDUAL OF FOURTEEN WATER SAMPLES FROM WATER SYSTEM I

	DAPI		ACRIDINE ORANGE			
Sample	Total Bacteria (Counts/ml)	DAPI/INT Positive Bacteria (Counts/ml)	Total Bacteria (Counts/ml)	AO/INT Positive Bacteria (Counts/ml)	Standard Plate Count (cfu/ml)	Chlorine Residual mg/l
1	1598	16	6918	46	0	0.9
2	806	52	2082	112	0.7	0.94
3	2218	80	3420	130	0	0.81
4	2408	118	5492	196	0.3	0.66
5	1000	10	2430	32	0	0.84
6	2398	1014	5198	1232	0	0.89
7	3534	2706	4236	2736	0.3	0.65
8	2054	566	3172	796	1	0.89
9	2422	824	4490	958	0.3	0.82
10	2308	1052	3050	1356	--	0.76
11	2708	54	3404	62	0.3	0.82
12	1998	10	2846	20	0	1.08
13	3446	82	5784	86	0	1.10
14	1190	6	2418	6	0	1.14

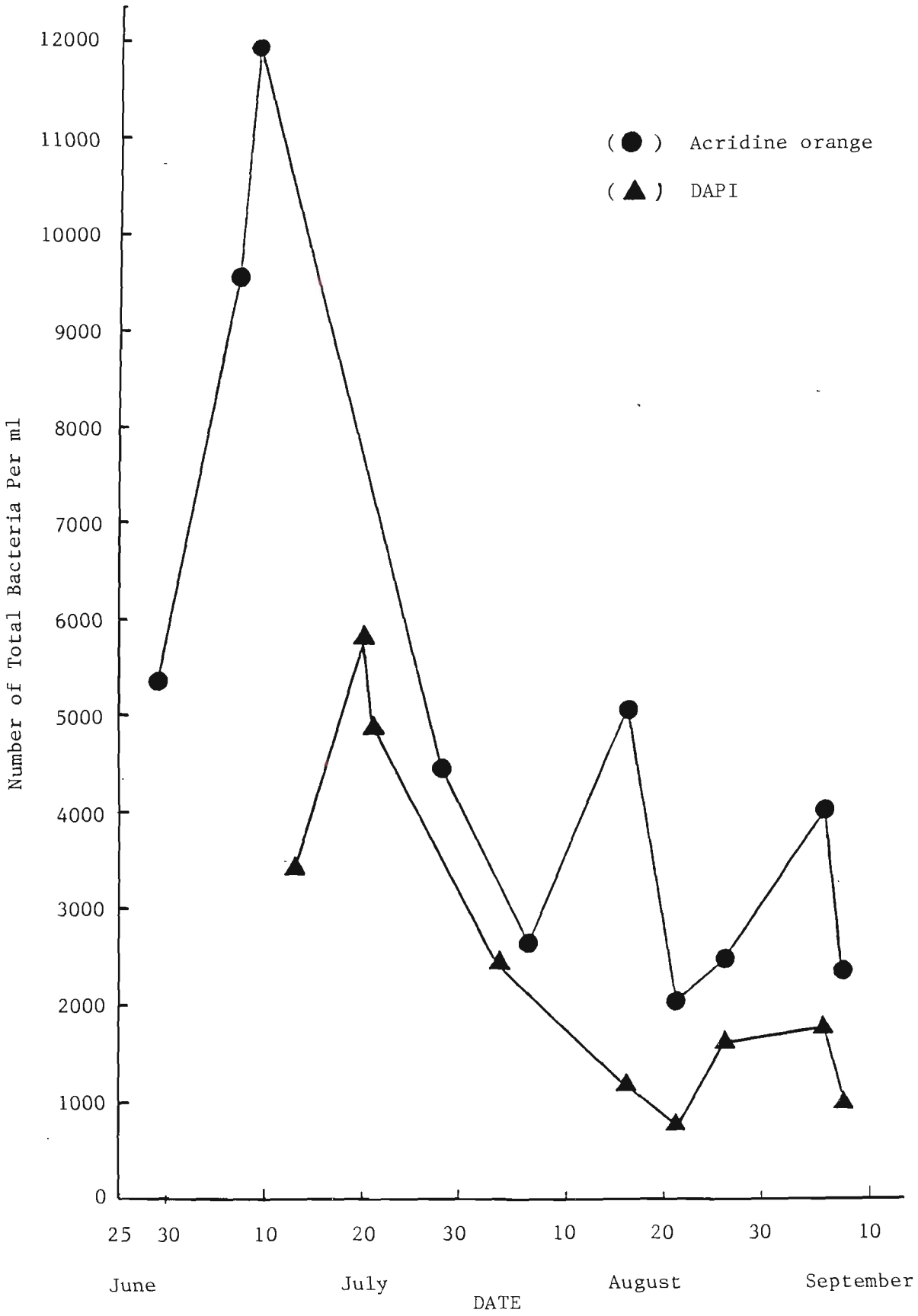

Figure 8. Total Bacterial Counts of Drinking Water Samples Taken from a Given Sampling Site at Various Sampling Dates

against which the maximum recovery of the total bacteria can be measured. DAPI is a highly specific fluorochrome for DNA and produce a stable bright blue fluorescence, whereas, acridine orange is a more general nucleic acid stain which produces green, orange-red and sometimes yellow fluorescence when complexed with bacterial cells.

The metabolically active bacterial cells were determined by a modified INT reduction method, and results are shown in Table IX. Data analysis indicated that potable water samples contained a wide range of metabolically active cells. For drinking water samples from the water system I as low as 0.25% to high 64.6% of the acridine orange treated samples and 0.51% to 76.6% of the DAPI treated cells were INT positive cells. This study indicated the average value of about 16% of total bacteria in potable water samples examined was metabolically active cells. In contrast,all 14 samples yielded less than 1.0 cfu per ml of water by standard plate count method.

For drinking water samples from the water system II the average value of about 6% of total bacteria had exhibited positive INT reduction; however, the metabolically active cells ranged from 900 to 18,000 counts per ml. The viable plate count analysis of the drinking water samples from the water system II ranged from 4.5 to 299 colony forming units per ml of water.

Results of the total number of fluorescent cells observed by both procedures, i.e., acridine orange and DAPI staining, were compatible. In general, drinking waters from the water system II exhibited higher counts than those from the water system I. The range total bacteria present in drinking waters of the water system II was 10^4 to 4.5×10^5 per ml as measured by epifluorescent direct count method.

E. Conclusions

This study evaluated both qualitative and quantitative analyses of microorganisms present in potable waters using two water supply systems. Because it would be an enormous task to enumerate all the bacteria in water, the study had focused on four areas. By utilizing the selective enrichment techniques major groups of predominant microbial population were determined. Only those organisms that directly or indirectly involved in microbiotic cycles of elements carbon, nitrogen, sulfur and iron were investigated. A variety of microorganisms were detected in various selective enrichment culture media. Isolation and identification of those organisms that grew on selective enrichment culture were the most difficult task since the majority of isolates that grew on primary isolation media simply did not grow further. Neither the conventional nor the commercial identification procedures were useful. It was assumed that many aquatic bacteria were nutritionally stressed environment and, therefore, they may have exhibited limited capabilities in various laboratory culture media. This study yielded an improved alternative technique for the enumeration of the most predominant group of bacteria, aerobic and facultative anaerobic heterotrophs. Enumeration of viable bacterial counts improved up to six fold when compared to the standard plate count procedure. This study further examined the quantitative analysis of microorganisms in drinking water by direct count method using fluorescent stains and epifluorescent microscopy techniques. The study indicated up to four orders of magnitude difference between viable count via standard plate count technique and epifluorescent total direct counts.

In summary, the microbial flora of potable waters can be determined by analyzing both qualitative and quantitative measurements. However, more studies are needed to improve the culturing techniques of the majority of organisms

observed in potable waters. Large discrepancies existed between bacterial counts, using direct count method and viable counts using plate count method. This may be due to our lack of knowledge in culturing dormant or metabolically inactive organisms which are present in nutritionally stressed habitat like drinking water.

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APPENDIX A

DESCRIPTION OF ISOLATES OBSERVED IN SELECTIVE ENRICHMENT CULTURE MEDIA

Colonial and Cellular Morphologies of Isolates	Sample Dates	Sample Locations 1	2	3	4	5	6	7
A. Iron Precipitating Bacteria								
a. Orange pigmented, umbonate colonies irregular form with undulate margins. Extremely tiny rods with a granular iron precipitate.	7-28-80	NA	+	+	+	NA	NA	NA
	8-11-80	-	+	+	+	+	+	NA
	8-19-80	+	+	+	+	+	+	+
	9-30-80	+	+	+	+	+	-	-
	10-17-80	-	-	-	-	-	-	-
b. Non-pigmented, white irregular colonies with undulate margins. Very tiny rods or cocci with a granular precipitate.	8-11-80	-	+	+	+	+	-	NA
	8-19-80	+	-	+	+	-	-	-
	9-30-80	-	+	+	-	-	+	+
	10-17-80	-	+	+	-	-	+	-
c. Yellow pigmented, umbonate colonies; irregular form with lobate margins. Moderate to small, thin, bipolar rods with an iron precipitate.	8-11-80	+	+	-	+	-	+	-
	8-19-80	+	+	-	-	-	-	-
	9-30-80	-	-	-	+	-	-	-
	10-17-80	-	-	-	+	-	+	-
d. Yellow pigmented, circular colonies with diffuse margins. Small fat, motile rods, with granular yellow precipitate.	8-11-80	-	+	-	-	-	+	NA
	8-19-80	-	-	-	+	-	-	-
	9-30-80	-	-	-	-	-	-	-
	10-17-80	-	+	-	-	-	-	-
e. Yellow pigmented, spindle shaped colonies with smooth, entire margins. Small motile rods with granular sheaths.	8-11-80	-	+	-	+	+	-	NA
	8-19-80	+	+	+	+	-	-	-
	9-30-80	-	+	-	+	+	-	NA
	10-17-80	-	-	-	-	-	-	-
f. Orange pigmented colonies, circular to spindle form with entire margins. Small, fat, motile rods with an iron ppt.	8-11-80	-	-	-	-	-	-	NA
	8-19-80	+	+	-	+	+	+	+
	9-30-80	-	-	-	-	-	-	-
	10-17-80	-	-	-	-	-	-	-
g. Orange pigmented, circular colonies with diffuse margins. Small, thin rods with granular sheaths.	8-11-80	-	-	-	-	-	-	NA
	8-19-80	-	-	-	-	-	-	-
	9-30-80	-	-	-	-	+	-	+
	10-17-80	-	-	+	-	+	+	-
h. Orange pigmented, filamentous to rhizoid surface growth. Extremely tiny rods or cocci with an iron ppt.	8-11-80	-	-	-	-	-	-	NA
	8-19-80	-	-	-	-	-	-	-
	9-30-80	+	-	+	-	-	-	-
	10-17-80	+	-	-	-	-	-	-

APPENDIX A (continued)

B. Filamentous Iron Bacteria

a. Non-pigmented, circular colonies with diffuse margins. Long thin, motile rods with filamentous extensions.	8-11-80	-	-	+	+	+	+	NA
	8-19-80	+	-	+	+	-	-	+
	9-30-80	+	+	+	+	+	+	+
	10-17-80	-	+	-	-	+	+	+
b. Non-pigmented, filamentous colonies. Large, clear filaments septate with no branching (possibly Leptothrix).	8-11-80	-	-	-	-	-	-	NA
	8-19-80	-	+	-	-	-	-	-
	9-30-80	-	-	-	-	-	-	-
	10-17-80	-	-	+	-	+	+	+

C. Sulfur Oxidizing Bacteria

a. Non-pigmented, white circular to spindle form colonies. Small rounded rods with associated yellowish precipitate.	7-28-80	NA	-	-	-	NA	NA	NA
	8-11-80	-	-	+	+	-	-	NA
	8-19-80	+	+	-	-	+	+	+
	9-30-80	+	+	+	+	+	+	-
	10-17-80	+	+	-	-	-	-	-
b. Yellow pigmented, circular colonies with diffuse to undulate margins. Very tiny rods or cocci, found in clusters with a surrounding white ppt.	8-11-80	+	+	-	+	-	+	NA
	8-19-80	-	+	+	+	+	+	-
	9-30-80	+	-	+	-	-	-	-
	10-17-80	-	-	-	-	-	+	+
c. Yellow pigmented colonies, circular form with entire margins. Very tiny rods with a yellowish-white precipitate.	8-11-80	-	+	-	-	-	+	NA
	8-19-80	-	-	-	+	+	+	+
	9-30-80	-	-	-	-	+	+	-
	10-17-80	-	-	-	-	-	-	-

APPENDIX A (continued)

D. Nitrogen Fixing Bacteria

a. Non-pigmented, circular colonies with diffuse margins. Tiny, bipolar rods found in clusters.	8-11-80	+	+	+	+	-	+	NA
	8-19-80	+	-	-	+	-	-	-
	9-30-80	+	+	-	+	+	+	+
	10-17-80	+	+	+	+	+	+	+
b. Pink pigmented, circular colonies with entire margins. Small, fat, motile rods.	7-28-80	NA	+	-	-	NA	NA	NA
	8-11-80	-	+	+	+	+	+	NA
	8-19-80	-	-	+	-	-	+	-
	9-30-80	+	+	-	-	+	+	+
	10-17-80	-	-	-	+	+	-	-
c. Yellow pigmented, circular to spindle form colonies with entire margins. Large fat rods.	8-11-80	+	+	+	+	-	-	NA
	8-18-80	+	-	+	+	+	+	+
	9-30-80	+	-	-	+	-	-	-
	10-17-80	-	-	+	+	-	-	+
d. Non-pigmented, irregular shaped colonies with undulate margins. Very tiny, bipolar rods.	8-11-80	-	-	-	+	+	-	NA
	8-19-80	+	-	+	-	+	-	-
	9-30-80	+	+	+	+	+	-	-
	10-17-80	+	-	+	-	+	-	+
e. Non-pigmented, spindle form colonies with entire margins. Long, thin, bipolar rods.	8-11-80	-	-	-	+	-	-	NA
	8-19-80	-	-	-	+	-	-	-
	9-30-80	+	+	+	-	-	+	-
	10-17-80	-	+	+	-	-	+	+
f. Pink pigmented, circular colonies with undulate margins. Large, fat, motile rods.	8-11-80	-	-	-	-	+	+	NA
	8-19-80	-	-	-	-	-	+	+
	9-30-80	+	+	+	-	-	+	-
	10-17-80	-	-	+	+	+	+	+

E. Stalked/Appendage-Producing Bacteria

a. Non-pigmented, punctiform to circular colonies with undulate margins. Moderate sized, fat, non-motile rods with an apparent attachment appendage; found in clusters.	7-28-80	NA	+	+	+	NA	NA	NA
	8-11-80	+	+	+	+	+	+	NA
	8-19-80	+	+	+	+	+	+	-
	9-30-80	-	+	+	-	-	+	-
	10-17-80	-	-	+	-	-	-	+
b. Non-pigmented, circular colonies with diffuse to erose margins. Small, fat, curved rods with a small extension at one end.	8-11-80	+	+	-	+	-	-	NA
	8-19-80	+	-	+	+	+	-	+
	9-30-80	+	-	-	-	+	+	+
	10-17-80	+	+	-	+	+	+	-
c. Pink pigmented, irregular shaped colonies with undulate margins. Large, fat rods with budding type growth.	7-28-80	NA	+	+	+	NA	NA	NA
	8-11-80	-	-	+	-	+	-	NA
	8-19-80	-	+	+	-	+	-	-
	9-30-80	-	+	-	-	-	+	-
	10-17-80	+	-	-	-	+	+	+
d. Pink pigmented, circular to spindle form colonies with entire margins. Short, fate rods, some with elongate extensions.	8-11-80	-	-	-	+	+	+	NA
	8-19-80	-	-	-	+	-	+	-
	9-30-80	-	+	+	-	-	+	-
	10-17-80	-	-	-	-	+	+	+
e. Yellow pigmented, punctiform colonies with entire to undulate margins. Tiny, fat, non-motile rods with a fiberous extension at one end, i.e., a holdfast.	7-28-80	NA	+	-	+	NA	NA	NA
	8-11-80	+	+	+	-	+	-	NA
	8-19-80	-	+	-	-	-	-	-
	9-30-80	-	-	+	+	+	-	-
	10-17-80	-	-	-	-	-	+	-
f. Yellow pigmented, circular colonies with diffuse to erose margins. Small, fat, non-motile rods with a holdfast extension at one end, tend to form aggregates and chains.	8-11-80	-	+	-	-	-	+	NA
	8-19-80	-	+	-	-	-	-	+
	9-30-80	+	-	+	+	-	-	-
	10-17-80	+	-	+	-	-	-	+
g. Yellow pigmented, spindle form colonies with entire margins. Small, long, thin, non-motile rods with a holdfast structure.	8-11-80	-	-	-	-	+	-	NA
	8-19-80	-	+	-	-	+	-	-
	9-30-80	+	-	-	-	+	-	-
	10-17-80	-	-	-	+	+	+	-
h. Orange pigmented, circular to spindle form colonies with entire margins. Long, thin rods with a holdfast extension at one end.	8-11-80	-	+	-	-	-	+	NA
	8-19-80	+	+	-	-	-	-	-
	9-30-80	-	-	-	-	+	-	-
	10-17-80	-	-	+	-	-	-	-
i. Non-pigmented, circular to spindle form with entire margins. Long, thin, vibrio shaped rods.	8-11-80	-	-	-	+	-	+	NA
	8-19-80	+	-	-	+	-	-	-
	9-30-80	+	-	-	-	-	-	+
	10-17-80	+	-	-	-	-	+	-

APPENDIX A (continued)

F. Actinomycetes

a. Non-pigmented, irregular form colonies with undulate margins. Long filaments with true branching and tiny bud-like out growths.	8-19-80	-	-	+	+	-	-	+
	9-30-80	+	-	-	-	+	+	-
	10-17-80	+	+	+	+	+	+	+
b. Non-pigmented, highly filamentous colonies. Large, true branching filaments with budding extensions.	8-19-80	-	-	-	-	-	-	-
	9-30-80	+	+	-	-	+	-	-
	10-17-80	+	+	+	+	-	+	-
c. Pink pigmented, circular to spindle form with entire margins. Moderate sized, bipolar rods with true branching.	8-19-80	-	+	+	-	+	+	+
	9-30-80	-	+	+	-	-	-	+
	10-17-80	-	-	-	-	-	-	+
d. Pink pigmented, circular colonies with erose to diffuse margins. Long, thin, clear filaments.	8-19-80	-	+	-	-	-	+	+
	9-30-80	-	+	-	-	+	-	-
	10-17-80	-	-	-	-	-	-	+
e. Yellow pigmented, circular colonies with undulate margins. Moderate sized, motile, bipolar rods, some develop into filamentous forms.	8-19-80	+	-	-	+	-	+	+
	9-30-80	-	-	+	+	-	-	-
	10-17-80	-	-	-	-	-	-	-

\+ positive growth
\- negative growth
NA not analyzed

APPENDIX B

Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
1	III	White	Rod	-	+	alk.	Tr	+	+	-	+	+
2	I	White	Rod	-	+	Alk.	+	+	-	-	+	-
3	II	Brown	Rod	v	-	NC	Tr	-	-	-	-	-
4	II	Yellow	Rod	-	+	Acid	Tr	+	-	+	+	-
5	II	Yellow	Rod	-	+	Alk.	Tr	+	-	+	+	+
6	III	Yellow	Rod	-	+	Alk.	Tr	+	-	+	+	+
7	II	White	Rod	+	-	Acid	Tr	-	-	-	-	+
8	IV	Yellow	Rod	-	-	Alk.	Tr	+	+	-	+	+
9	IV	Yellow	Rod	-	-	Acid	Tr	+	-	-	+	-
10	I	White	Rod	-	-	Alk	+	+	+	+	+	+
11	I	White	Rod	-	-	Acid	Tr	-	-	-	-	-
12	I	White	Rod	-	-	NC	-	+	+	+	+	-
13	II	Yellow	Rod	-	-	Alk	+	-	-	-	+	+
14	III	White	Rod	v	-	NC	Tr	+	-	-	-	-
15	II	White	Rod	+	+	Alk.	+	+	-	-	+	+

I - Modified Winogradsky's iron precipitating medium
II - Stoke's medium
III - Thiosulfate mineral medium
IV - Nitrogen-free medium
V - Basal mineral medium with nitrate
VI - AGS-actinomycetes medium
VII - R2A plate count medium

APPENDIX B (Continued)
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Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
16	II	White	Rod	-	+	Alk.	+	+	-	-	+	+
17	I	White	Rod	-	+	Alk.	+	-	-	-	+	+
18	III	White	Rod	-	+	Alk.	Tr	-	-	-	+	+
19	III	Yellow	Rod	-	-	Alk.	+	+	+	-	+	+
20	III	White	Rod	-	+	Alk.	+	-	-	-	+	+
21	VI	Orange	Rod	-	-	Acid	-	+	+	+	+	+
22	VI	Yellow	Rod	-	+	Alk.	Tr	+	-	+	+	+
23	VI	White	Rod	-	+	Alk.	+	+	-	-	+	-
24	VI	Yellow	Rod	-	+	Acid	+	+	+	-	+	+
25	VI	White	Rod	+	+	Alk.	+	-	-	-	+	+
26	II	Yellow	Rod	-	+	Acid	+	+	+	-	+	+
27	IV	White	Rod	-	-	Alk.	Tr	-	-	+	+	+
28	I	Orange	Rod	+	+	Alk.	Tr	-	-	-	+	+
29	VI	Brown	Rod	-	+	Alk.	+	+	+	+	+	+
30	VI	Pink	Rod	-	-	Alk.	Tr	-	-	+	+	-

APPENDIX B (Continued)
Page 3

Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
31	VI	Yellow	Rod	-	+	Alk.	+	-	-	+	+	-
32	VI	White	Rod	-	+	Alk.	+	-	-	-	+	-
33	VI	Yellow	Rod	-	+	Alk.	+	+	+	+	-	+
34	V	White	Rod	-	-	Acid	+	-	-	-	+	-
35	IV	White	Rod	-	+	Alk.	+	+	+	-	+	+
36	IV	Yellow	Rod	-	+	Alk.	Tr	-	-	-	+	+
37	II	Yellow	Rod	-	-	Acid	Tr	-	-	+	+	-
38	VI	White	Rod	-	+	Alk.	+	-	-	-	+	+
39	I	Orange	Rod	-	+	Alk.	+	-	-	-	+	+
40	VI	White	Rod	-	+	Alk.	+	-	-	-	+	-
41	II	Yellow	Rod	-	+	Alk.	Tr	-	-	-	+	+
42	IV	Beige	Rod	-	+	Acid	Tr	+	-	+	+	-
43	VI	Brown	Cocci	+	-	Alk.	-	+	+	+	+	-
44	I	Yellow	Rod	-	+	Alk.	Tr	-	-	+	+	+
45	I	Pink	Rod	-	-	Alk.	Tr	-	-	-	+	-

APPENDIX B (Continued)
Page 4

Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
46	II	Beige	Rod	-	+	Alk.	-	+	+	+	+	+
47	II	White	Rod	-	-	NC	-	-	-	-	+	-
48	VI	White	Rod	-	+	Alk.	+	+	+	-	+	-
49	II	White	Rod	-	-	Acid	-	-	-	+	+	+
50	I	Beige	Rod	-	+	Alk.	+	-	-	+	+	+
51	II	White	Rod	-	+	Alk.	+	-	-	-	+	+
52	VI	White	Rod	-	+	Alk.	+	-	-	-	+	+
53	VI	Pink	Rod	-	+	Alk.	+	-	-	-	+	-
54	VI	Orange	Cocci	-	-	Alk.	+	-	-	-	+	-
55	VI	Yellow	Rod	-	-	Acid	Tr	-	-	-	+	+
56	II	Yellow	Rod	-	+	Alk.	-	+	+	+	+	+
57	IV	Brown	Rod	-	+	Alk.	+	-	-	-	+	-
58	II	Beige	Rod	-	+	Alk.	+	-	-	-	+	+
59	VI	Beige	Rod	-	+	Alk.	+	-	-	-	+	-
60	II	Orange	Rod	-	+	Alk.	Tr	-	-	-	+	-

APPENDIX B (Continued)
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Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
61	II	White	Rod	-	+	Alk.	Tr	-	-	-	+	-
62	V	White	Rod	-	+	Alk.	Tr	-	-	-	+	-
63	V	White	Rod	-	-	NC	-	-	-	-	-	-
64	V	White	Rod	-	+	Alk.	Tr	-	-	-	+	-
65	I	White	Rod	-	-	Acid	-	-	-	+	+	-
66	I	White	Rod	-	+	NC	-	-	-	+	+	-
67	I	Beige	Rod	-	+	Alk.	-	+	+	+	+	+
68	I	White	Rod	-	+	NC	-	+	+	+	+	-
69	I	White	Rod	-	+	NC	-	-	-	+	+	-
70	I	White	Rod	-	+	NC	-	-	-	-	+	-
71	I	Orange	Rod	-	+	Alk.	Tr	-	-	-	+	-
72	I	White	Rod	-	+	NC	-	-	-	-	+	-
73	V	White	Rod	-	+	Alk.	+	+	+	-	+	-
74	V	Pink	Rod	-	+	NC	-	-	-	+	+	-
75	II	White	Rod	-	+	Alk.	+	-	-	-	+	-

APPENDIX B (Continued)
Page 6

Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
76	II	White	Cocci	-	+	Acid	+	+	+	-	+	+
77	VII	Orange	Rod	-	+	Acid	-	+	-	+	+	+
78	VII	White	Rod	-	+	Alk.	Tr	-	-	-	+	+
79	VII	White	Rod	-	+	Alk.	+	+	+	-	+	-
80	VII	White	Rod	-	+	NC	-	-	-	-	+	-
81	VII	Brown	Rod	-	+	NC	Tr	-	-	-	+	-
82	VII	White	Rod	-	+	NC	Tr	-	-	-	+	+
83	VII	White	Rod	-	+	Alk.	+	-	-	-	+	-
84	VII	White	Rod	+	+	Acid	Tr	-	-	-	+	-
85	VII	Orange	Rod	+	+	NC	Tr	-	-	-	+	-
86	VII	Pink	Rod	-	+	NC	-	-	-	+	+	-
87	V	White	Rod	+	+	NC	Tr	-	-	-	+	-
88	V	White	Rod	-	+	NC	Tr	-	-	-	+	-
89	V	Orange	Rod	+	+	NC	+	-	-	-	+	-
90	V	White	Rod	-	+	Alk.	+	-	-	-	-	-

APPENDIX B (Continued)
Page 7

Isolate No.	Primary Isolation Media	Growth on R2A Medium	Cellular Morphology	Gram Reaction	Growth on MacConkey Agar	Reaction in Triple Sugar Iron Agar		OF Basal Glucose Medium		Oxidase Reaction	Catalase Reaction	Motility
						Slant	H_2S Prod.	Oxidative	Fermentative			
91	V	White	Rod	v	-	NC	-	-	-	-	-	-
92	VI	Orange	Cocci	-	-	NC	-	-	-	-	-	-
93	VI	White	Rod	-	+	NC	-	-	-	-	+	+
94	VI	White	Rod	-	+	NC	-	-	-	+	+	-
95	VI	White	Rod	-	+	NC	Tr	+	+	+	+	-
96	VI	White	Rod	-	+	Alk.	Tr	-	-	-	+	-
97	VI	White	Rod	-	+	NC	-	-	-	-	+	-
98	I	White	Rod	-	+	NC	-	+	+	+	+	-

Motility:
Gram reaction: + = positive, - = negative, v = variable
Oxidase reaction:
Catalase reaction:

MacConkey agar: + = growth, - = no growth
OF basal glucose medium:

Triple sugar iron agar: Alk. = alkaline reaction
Acid = acid reaction
NC = no change
H_2S Production: Tr = trace amount
+ = positive production
- = negative production